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STUDIES ON THE EFFECTS OF INTERFERON ON THE  
PHENOTYPE OF MOUSE FIBROBLASTS THAT HAVE BEEN  
TRANSFORMED BY A MURINE SARCOMA VIRUS

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Presented for the degree of

Doctor of Philosophy

at the University of Warwick

February 1981.

The research was conducted at the  
Department of Biological Sciences,  
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LIST OF CONTENTS

	<u>Page no.</u>
INTRODUCTION	1
Part 1 -	
Transformation - An <u>In Vitro</u> Model for Malignancy.	4
Section A -	
Tissue Culture in the Study of Malignancy.	4
Establishment of Cells in Culture.	4
<u>In Vitro</u> Transformation.	7
Agents used for Transformation	7
Section B -	
The Transformed Phenotype.	11
Contact Inhibition.	11
Density Dependent Inhibition of Growth.	12
Anchorage Dependence.	14
Morphology and Cytoskeleton.	17
Cell Surface Components.	18
Increased Agglutinability.	21
Induction of Cell Surface Antigens.	23
Increased Nutrient Uptake	23
Secretion	24
Cyclic AMP	25
Correlation of <u>In Vitro</u> Transformation with <u>In Vivo</u> Malignancy	26



	<u>Page No.</u>
<u>In Vivo</u> Tumourigenicity	28
Section C -	
RNA Tumour Viruses - Tools for	
Transformation.	29
Relationship Between Mammalian Sarcoma	
and Lymphatic Leukaemia Viruses.	31
Structure of the Genome.	32
Src Gene and Transformation by RSV.	33
Transforming Genes of Murine Sarcoma	
Viruses.	35
Part 2 -	
Interferon's Potential as an Antitumour	
Agent.	37
Section A -	
Historical Perspective.	37
Section B -	
Properties of Interferon.	39
Types of Interferon.	39
Some Characteristics.	42
Section C -	
Production and Purification.	43
Inducers.	43
Yields.	44

	<u>Page No.</u>
Purification.	45
Section D -	
Cellular Actions of Interferon.	46
Antitumour Activities <u>In Vivo</u> .	46
Spontaneous Tumours.	48
Inhibition of Growth of Normal Cells	
<u>In Vivo</u> .	49
Clinical Trials.	49
Mechanisms of Antitumour Activity.	50
Inhibition of Growth <u>In Vitro</u> .	53
Growth Inhibition Studied in Synchronised	
Cultures.	56
Other Effects on the Cellular Phenotype.	57
Immune Effects of Interferon.	61
Possible Mechanisms for Interferon's	
Cellular Activities.	63
Reversion of the Transformed Phenotype.	69
<u>MATERIALS AND METHODS</u>	72
Cells.	75
Interferon Production and Purification.	76
Assay of Interferon Titres.	78
Measurement of Protein Content.	78
Measurement of Antiviral and Anticellular	
Activities in Different Clones.	80

Page no.

## DNA Synthesis at Different Cell

Densities.	80
Growth Curves and Saturation Density.	81
Focus Assay.	84
Anchorage Dependence.	82
Cloning Efficiency in Liquid Medium.	82
Concanavalin A Agglutination.	83
Morphological Studies.	83
Conjugation of FITC to DNase I.	85
Coupling of Affinity Adsorbents to CNBr-	
Activated Sepharose 4B.	86
Fibronectin Purification.	87
Preparation of Antiserum to Fibronectin.	88
Attachment of Cells to Glass.	88
Polyacrylamide Gel Electrophoresis of	
Cell Lysates.	89
Immunoprecipitation of p21.	90

RESULTS 92

## Chapter One

Production and Purification of Interferon.	92
A. Production of Interferon.	93
B. Purification.	95
C. Stability.	108
D. Discussion.	109

Page no.

## Chapter Two

Effects of Interferon on Growth of Cells.	112
A. Sensitivities to Antiviral and Cell Growth Inhibitory Activities.	112
B. Growth and Saturation Densities.	118
C. Effects of Cell Density on Interferon's Inhibition of DNA Synthesis.	128
D. Focus Formation.	130
E. Anchorage Independence.	131
F. Cloning Efficiency in Liquid Medium.	134
G. Effects of Serum Concentration on Interferon Activity.	135
H. Conclusions.	138

## Chapter Three

Interactions Between Butyric Acid and Interferon in Growth Inhibition.	140
A. Effects of Butyric Acid on Growth Curves.	140
B. Effects of Butyric Acid on <sup>3</sup> H-Thymidine Incorporation into Cellular DNA.	142
C. Conclusions.	145

## Chapter Four

Effects on Cell Surfaces and Morphology.	146
A. Agglutinability by Concanavalin A.	146
B. Morphology.	148

Page no.

## Chapter Two

Effects of Interferon on Growth of Cells.	112
A. Sensitivities to Antiviral and Cell Growth Inhibitory Activities.	112
B. Growth and Saturation Densities.	118
C. Effects of Cell Density on Interferon's Inhibition of DNA Synthesis.	128
D. Focus Formation.	130
E. Anchorage Independence.	131
F. Cloning Efficiency in Liquid Medium.	134
G. Effects of Serum Concentration on Interferon Activity.	135
H. Conclusions.	138

## Chapter Three

Interactions Between Butyric Acid and Interferon in Growth Inhibition.	140
A. Effects of Butyric Acid on Growth Curves.	140
B. Effects of Butyric Acid on <sup>3</sup> H-Thymidine Incorporation into Cellular DNA.	142
C. Conclusions.	145

## Chapter Four

Effects on Cell Surfaces and Morphology.	146
A. Agglutinability by Concanavalin A.	146
B. Morphology.	148

C. Microfilament System.	158
D. Cell Surface Fibronectin.	163
E. Adhesion to Glass Surfaces.	172
F. Polyacrylamide Gel Electrophoresis.	174
G. Conclusions.	176
 Chapter Five	
Effects of Interferon on p21 Levels.	179
A. Electrophoresis.	179
B. Quantitation.	182
 <u>DISCUSSION</u>	184
Part I Use of Interferon.	184
1. Interferon Purity.	184
2. Interferon Doses.	185
Part II The Effects of Interferon	187
1. Antiviral and Cell Growth Inhibitory Activities.	187
2. Growth and Saturation Densities.	189
3. Transformation-Specific Growth Parameters.	197
4. Agglutinability.	202
5. Adhesiveness.	203
6. Morphology and Fluorescence.	203
7. Effects of Interferon p21 Levels.	214
Part III General Conclusions.	216
 <u>REFERENCES.</u>	220

LIST OF TABLES

	<u>Page no.</u>
1. Purification of Interferon by Affinity Chromatography on BSA-Sepharose 4B (Experiment 1).	98
2. Purification of Interferon by Affinity Chromatography on BSA-Sepharose 4B (Experiment 2).	100
3. Purification of Interferon by Affinity Chromatography on Affi-Gel 202.	103
4. Purification of Interferon by Affinity Chromatography on Poly U-Sepharose 4B.	106
5. Interferon Doses which cause 50% Inhibition of Cell Growth and Virus Replication.	117
6. Effect of Interferon on DNA Synthesis at Different Cell Densities.	129
7. Effect of Interferon on Cloning Efficiency in Liquid, on Focus Formation and on Growth in Soft Agar of Two Normal and Six MSV- Transformed Cell Clones.	132
8. Effects of Serum Content of Medium on Interferon's Inhibition of Cloning on Plastic.	136

9. Agglutinability of CC1 and NIH 3T3 Cells  
by Con A after Growth in the Presence or  
Absence of Interferon. 147
10. Comparison of p21 Levels in Interferon-  
Treated and Control Cell Cultures. 183



LIST OF FIGURES

	<u>Page no.</u>
1. Purification of Interferon by Affinity Chromatography on BSA-Sepharose 4B (Experiment 1).	99
2. Purification of Interferon by Affinity Chromatography on BSA-Sepharose 4B (Experiment 2).	101
3. Purification of Interferon by Affinity Chromatography on Affi-Gel 202.	104
4. Purification of Interferon by Affinity Chromatography on Poly U-Sepharose 4B.	107
5. Sensitivity of Cell Lines to Interferon's Antiviral Activity.	113-114
6. Sensitivity of Cell Lines to the Growth Inhibitory Activity of Interferon.	115-116
7. Effects of Interferon on Growth and Saturation Densities of Several Cell Lines.	119-121
8. Effect of Interferon on Growth of Late Passage (30th passage) C3H10T $\frac{1}{2}$ Cells.	125
9. Lack of Effect of Mouse Interferon on Growth of Heterologous (Rat) Cells.	127

Page no.

10. Effect of Interferon on Growth and Saturation Densities of Cells Grown in Medium Containing a Very High Serum Content.	137
11. Effects of Butyric Acid, in the Presence or Absence of Interferon, on the Growth of Cells.	141
12. Effect of Interferon and Butyric Acid on DNA Synthesis of Cells at Different Densities.	143-144
13. The Effects of Interferon and Butyric Acid on Cell and Culture Morphology.	150-156
14. The Effects of Interferon on the Micro-filament System of Normal and Transformed Cells.	160-162
15. Effects of Interferon and Butyric Acid on Fibronectin Distribution.	165-168
16. Effects of Interferon and Butyric Acid on Distribution of Fibronectin in Sub-cellular Matrix.	169-170
17. Ability of Cells Grown in Interferon to Adsorb to Glass.	173

18. SDS Polyacrylamide Gel Electrophoresis  
of Whole Cell Lysates Taken from Cells  
Grown with or without Interferon ( $10^4$  U/ml)  
and/or Butyric Acid (0.5mM), and Labelled  
with  $^{35}$ S-methionine. 175
19. Levels of Actin in Cells Treated with  
Butyric Acid and Interferon for up to  
One Week. 177
20. Levels of Fibronectin in Cells Treated  
with Butyric Acid and Interferon. 178
21. SDS Polyacrylamide Gel Electrophoresis of  
Cell Lysates Precipitated with Antisera  
to p21. 180-181

Acknowledgements

I wish to thank those many people who have been involved with my project, particularly my supervisors, Professor D. C. Burke and Dr. A. G. Morris, for their endless discussion and criticism of my work. I am also indebted to innumerable people within the department for their frequent advice, assistance and use of equipment, especially Mr. R. Bird for maintaining a constant supply of interferon, Mrs. B. Wood for maintaining a constant flow of growth medium, Dr. F. Cooke for conducting the immunoprecipitation experiments and Dr. K. Logan for instruction in the use of the Reichert ultraviolet fluorescent microscope. Dr. D. Bray and his colleagues of the MRC Biophysics unit, Kings' College, London, gave me valuable instruction in immunofluorescence techniques and , Dr. R. C. Hughes of the National Institute of Medical Research, Mill Hill, London, gave me advice and donated a small amount of antiserum to fibronectin. Finally I must thank Miss C. Alderson for typing the final version of this thesis.

This work was undertaken by award from the Cancer Research Campaign.

Declaration

Work from this thesis is presently in press with the Journal of Cell Science, and is reported herein in Chapter 2 and the Morphology and Microfilament System sections of Chapter 4. This work and all other studies in this thesis were conducted by myself except for the studies on p21 levels in transformed cells which were conducted jointly between myself and Dr. F. Cooke of this Department.

LIST OF ABBREVIATIONS

ALS	Antilymphocyte Serum
ALV	Acute Leukaemia Virus
AMD	Actinomycin D
ASV	Avian Sarcoma Virus
ATP	Adenosine Triphosphate
BA	Butyric Acid
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine-3',5'-monophosphate
CAPS	Cyclohexylaminopropanesulphonic Acid
cDNA	Complementary Deoxyribonucleic Acid
CEA	Carcinoembryonic Antigen
Ci	Curie
cl	Clone
CNBr	Cyanogen Bromide
CO <sub>2</sub>	Carbon Dioxide
Con A	Concanavalin A
cpm	Counts per minute
CSA	Cell Surface Antigen
cyclic GMP	Cyclic Guanosine-3', 5'-Monophosphate
DMEM	Dulbecco's Modification of Eagle's Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DNase I	Deoxyribonuclease I
dsRNA	Double-Stranded Ribonucleic Acid
EA	Ehrlich Ascites

EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
eIF2	Eukaryotic protein synthesis Initiation Factor 2
EMC	Encephalomyocarditis Virus
FITC	Flourescein Isothyocyanate
GI50	Interferon dose giving 50% inhibition of DNA synthesis
GMEM	Glasgow's Modification of Eagle's Medium
HaSV	Harvey Sarcoma Virus
HAU	Haemagglutinating Units
HCl	Hydrochloric Acid
IFN	Interferon
IUdR	Iododeoxyuridine
KiLV	Kirsten Leukaemia Virus
KiSV	Kirsten Sarcoma Virus
KNRK	Normal Rat Kidney Cells transformed by KiSV
LD50	A dose (of a substance, tumour cells etc.) that will kill half of its target (cells, animals etc.)
LETS	Large External Transformation-Sensitive Protein
LLV	Lymphatic Leukaemia Virus
MEF	Mouse Embryo Fibroblasts
MEV	Mouse Erythroblastosis Virus
met.tRNA.40s	Methionyl-transfer ribonucleic acid-40s ribosomal complex

MoLV	Moloney Leukaemia Virus
MoSV	Moloney Sarcoma Virus
MSV	Murine Sarcoma Virus
NaCl	Sodium Chloride
$\text{Na}_2\text{CO}_3$	Sodium Carbonate
$\text{NaHCO}_3$	Sodium Hydrogencarbonate
$\text{NaOH}$	Sodium Hydroxide
NDV	Newcastle Disease Virus
$^{63}\text{Ni}$	Nickel-63
NIH	National Institutes of Health
NK	Natural Killer
NRK	Normal Rat Kidney
PA	Plasminogen Activator
pH	$-\log_{10}$ hydrogen ion concentration
PHA	Phytohaemagglutinin
Poly(HEMA)	Poly(2-Hydroxyethylmethacrylate)
Poly rI. poly rC.	Polyribonucleosinic Acid. polyribocytidylic acid
Poly U	Polyuridylic acid
POPOP	1, 4-di(2(5-phenyl-oxazolyl))-benzene
pppA2'p5'Ap5'A. (2.5A).	5'-Triphospho-2', 5'-Oligoadenylic acid
RSV	Rous Sarcoma Virus
SDS	Sodium Dodecyl Sulphate
SFV	Semliki Forest Virus
SMA	Surface Modulating Assembly



SRBC	Sheep Red Blood Cells
TEMED	N, N, N', N'-Tetramethylethylenediamine
VI50	Interferon dose that inhibits virus replication by 50%
VSV	Vesicular Stomatitis Virus
WGA	Wheatgerm Agglutinin

### SUMMARY

The aim of this research was to establish whether or not mouse interferon could reverse the phenotype of transformed cells so that they behaved in a more normal manner.

For this study, clonal isolates of transformed cells from two continuous cell lines and fibroblasts extracted from mouse embryos were used.

It was found that interferon could inhibit the growth of both normal and transformed cells. With several transformed clones interferon also reduced their saturation densities, which were normally several fold higher than those of the non-transformed parents. This suggested that interferon had induced a partial reversion to density-dependent growth control. Butyric acid also inhibited growth rate and acted additively with interferon when cells were treated with the two agents together.

Interferon had a variable effect on the ability of dispersed cells to form colonies on plastic substrate in liquid media, but had a consistently greater effect on the ability of transformed cells to form foci on a monolayer of normal cells, and to grow suspended in agar, two growth conditions specific to the transformed state. It was concluded that interferon had inhibited focus formation and growth in agar by a combination of its growth inhibitory activity and an effect specific for the transformed phenotype.

Interferon also affected the morphology of both normal and transformed cells. The cells became more spread-out, and in transformed cells there was a partial restoration of the microfilament bundle system. Despite these effects on the cytoskeleton, the extracellular matrix of fibronectin fibrils appeared to be little altered by interferon, except when added in conjunction with butyric acid. Under these circumstances, the fibronectin matrix became much more extensive.

These data increase the likelihood that interferon's in vivo antitumour activity involved a direct effect on the tumour cells themselves, such that these cells behave more normally.

## INTRODUCTION

In the Western World cancer is a major disease. For a number of years research has been conducted in order to understand the mechanisms of tumour formation, to learn how to detect and prevent the processes which lead to a tumour, and to find methods to cure patients in which tumours have already grown.

At present a number of cancers can be treated successfully by a range of techniques employing combinations of chemotherapy, radiotherapy and surgery, though such treatments can be toxic to the patient.

Interferon was originally identified as an antiviral agent, but in recent years has been shown to possess considerable potential for the treatment of a range of tumours, possibly with minimal side-effects. Experiments with animals, mainly mice, found that it could prevent the growth of transplanted tumours and has also caused some well-developed tumours to regress. Trials with people, however, have barely begun, having been used only in a few isolated cases, using small numbers of patients.

Full-scale clinical trials are now being planned both in Europe and the U.S.A., but one of the major problems facing these trials is how to secure a sufficient supply of interferon. The past eighteen months have seen

some dramatic advances towards improving the supply, which, coupled with the planned trials and treatment of two young patients in Glasgow, has resulted in considerable publicity in the media.

To date almost all the interferon used in tests on people has been produced in batch processes by induction of leucocytes or lymphoblastoid cells grown in suspension. Such techniques are time-consuming, expensive and generate relatively small amounts of interferon. Genetic manipulation of human interferon genes into bacteria has resulted in isolation of clones which constitutively produce quite large amounts of interferon. This material has yet to be fully characterised and it is not known if it will be effective in humans. If it is found to be active it should then become possible to continuously produce vast quantities on a large industrial scale, thus overcoming the problems of costs and supply for clinical application.

These developments have placed interferon firmly in the vanguard of the 'revolution' in biotechnology and, if successful, may point the way towards enabling large-scale production of other biological agents for important medical, agricultural and environmental applications.

Before interferon can be successfully employed as an anti-cancer agent it is important to establish the parameters within which it will be effective, such as

dosage, duration of treatment, mode of action, specificity for tumours and what might result from effects on growth and behaviour of normal tissues. Some of these can only be established through the clinical trials, but others, such as mode of action and effects on normal tissues can be studied in the laboratory. This thesis was initiated, as part of a programme in this laboratory, to study the ways in which interferon affects the transformed phenotype, and establish whether interferon can actually revert the transformed phenotype towards a more normal behaviour.

The following introduction is divided into two parts. The first discusses how the application of tissue culture has advanced our understanding of neoplasia over the past few years, and how in vitro transformation can be correlated with in vivo malignancy. The second part introduces interferon and surveys our present knowledge of its anti-cellular and antitumour actions.

## PART I

### TRANSFORMATION - AN IN VITRO MODEL FOR MALIGNANCY

#### Section A Tissue Culture in the Study of Malignancy

The study of a range of tumour explants and normal cells which have been transformed in vitro has significantly advanced our understanding of the controls placed upon the growth of normal cells and of the process whereby cells free themselves from these controls to become tumourigenic (4, 128, 189, 197, 204). However, tissue culture does have a number of limitations that stem from the way in which cells of different species and different tissue origin behave in widely differing fashions. This leads one to question how closely the properties of cells in culture reflect those prevalent in vivo. This problem of how relevant in vitro studies are to in vivo malignancy will be considered later. This section will discuss the basic properties and behaviour of different cell types grown in vitro, with a view to establishing which criteria need to be considered when selecting a cell system to study in vitro transformation.

#### 1. Establishment of Cells in Vitro

The most direct way to study tumourigenic cells in vitro is to explant human or animal tumours and establish these in tissue culture. Usually they grow poorly, and

their use suffers from a number of disadvantages which are at least partly due to most tumours being a heterogeneous collection of different cell types (204). One particular cell type may grow more vigorously than any of the others, and thereby come to dominate the culture, making it unrepresentative of the original tumour (ref 204 p403), and indeed may not even be the malignant cell type. Moreover, rodent cells, for reasons discussed below, when in vitro often spontaneously undergo changes which may greatly alter their characteristics (ref 204 p403). Finally, in a heterogeneous tumour it may be impossible to determine from which cell type the tumour originated and so there can be no normal cell type with whose behaviour it can be compared.

A more closely controlled experimental system can be established in vitro by explanting normal adult or embryonic tissues and then transforming these. The transformation process yields transformed cells whose properties can be directly compared with the normal parental line.

Apart from being diploid normal fibroblasts when explanted possess a number of characteristic behavioural properties;

1. they show contact inhibition of movement, i.e. migration of a cell in one particular direction is halted when contact is made with another cell, but movement in other directions is unhindered (3, 4).
2. their growth shows density dependent inhibition, i.e. when a confluent monolayer of cells is formed further

growth is arrested (158, 241, ref 115 p451).

3. their growth is anchorage dependent, i.e. they can only grow when attached to a solid substrate, not in suspension (242, ref 115 p452-3).

These properties will be discussed in greater detail later.

Cells of human and avian origin retain these properties for between ten and over 100 cell doublings, after which time the cultures die out (ref 204 p402). Such cells are said to be stable, and possess finite growth potential (204).

Cell cultures of rodent origin, however, frequently produce cultures which are aneuploid and have acquired infinite growth potential (ref 204, pp 402 & 409) - they will grow in culture forever. These cells are said to be of unstable species origin (204). The resultant aneuploid cultures invariably eventually transform spontaneously, and become tumourigenic in vivo (ref 204, pp402 & 408). However, it is possible to isolate by careful culture techniques lines which retain the phenotype of normal cells, even though they are aneuploid.

In this way the established 'normal' cell lines have been developed, such as the mouse 3T3's (2, ref 204 p409) and C3H 10T $\frac{1}{2}$  (210) and the baby hamster kidney (BHK) cells (240). These too tend to undergo spontaneous transformation after prolonged passage in culture (1, 204).



## 2. In Vitro Transformation

Ponten has defined transformation as the acquisition of permanent disturbance of growth and/or locomotion control (204). Thus, transformed cells tend to lose density-dependent growth (241, ref 115 p451, ref 204 pp 406 & 409), contact inhibition of movement (3, 4, ref 204 pp405-6) and anchorage dependence (242, ref 115 pp 452-3). The final and most fundamental criterion for transformation, however, is tumourigenicity in vivo. The correlation between in vitro transformation and in vivo tumourigenicity is not total, a problem that will be discussed later.

## 3. Agents used for Transformation

Agents which have been used for in vitro transformation are all known to cause tumours in vivo. They fall into three main categories;

- 1) Ultraviolet or X-ray irradiation (50, ref 204 p410),
- 2) Chemicals such as polycyclic aromatic hydrocarbons, alkylating agents and nitrosamides (62, 117) .
- 3) Oncogenic viruses, such as the DNA viruses SV40 and polyoma, plus the RNA tumour viruses, or retroviruses (115, 204) .

Chemical and radiation-induced transformation probably occurs by mutagenesis. In vivo many carcinogenic chemicals must be activated by oxidases. When activated such chemicals bind quite strongly to DNA and RNA (62, 117) in

target cells, and it is possible to detect chromosome alterations a few cell doublings after treatment (20, 21, 181).

Onset of transformation, however, can vary from just a few up to 80 or more cell doublings following treatment. Rodent cell lines have proved to be the easiest to transform by chemicals and radiation, but only quite recently has it been possible to transform human cells (140, 148, 211).

It has been suggested that resistance to chemical and radiation-induced transformation by human cells may be due to a highly efficient DNA-repair mechanism (62).

Oncogenic viruses transform cells by introducing new genes into the cellular genome which code for transformation. The whole process of infection and transformation is quite rapid and can be completed within a few cell doublings (204). Retroviruses will readily transform murine and avian cells, but not human, for which SV40 is the usual transforming agent (251-3). Maintenance of the transformed phenotype requires the constant presence of the product(s) of the transforming gene. Studies with mutants of Avian Sarcoma Virus (ASV) and Kirsten Murine Sarcoma Virus (KiSV) temperature sensitive for transformation have found that transformed cells rapidly revert to normal upon shift from the permissive to the non-permissive temperature (228, 232, ref 115 p423), thus suggesting rapid inactivation of the transforming gene's product(s).

The possibility that carcinogenic chemicals may transform

cells by activating endogenous oncornaviruses has been discounted since no viral synthesis can be detected in chemically transformed cells, whilst agents which do activate endogenous viruses, such as iododeoxyuridine (IUDR), do not transform cells (117).

The tendency for an established cell line to continue through a series of changes, progressing towards a more transformed state, from which clones of spontaneous transformants can frequently be isolated (204), creates problems when comparing the behaviour of transformed clones with the 'normal' parental line, since the 'normal' line may itself be progressively becoming more transformed.

The use of cells of stable species (i.e. human and avian) may overcome these problems since their behaviour remains constant throughout and a culture of normal cells is more likely to be representative of its in vivo ancestors.

The study of human cancer should ideally use human cells in vitro, but, as already described, transformation of human cells by chemical carcinogens and radiation has only recently been achieved. Transformation of human cells by retroviruses is also difficult, and the only really successful agent routinely used is SV40 (234, ref 253 pp360-1). The ease with which avian and rodent cells transform with retroviruses has made these cells the choice system for many studies despite the problems of instability.

It could be argued that the study of viral transformation is irrelevant to human cancer since no human tumour, with the exception of papillomas, has yet been found to be virus-induced. This argument is strengthened by three points:-

- a) The initial stages of transformation by chemicals (the main human carcinogens) are different from those with oncogenic viruses,
- b) The latent period before transformation by oncogenic viruses is complete is only a few days, but can be up to several weeks for chemicals (20, 21, 53, 140, 181, 211),
- c) Some human tumour explants may still show some normal growth and movement control which is only lost when the cells are infected and transformed by oncogenic viruses (ref 204 p411).

On the other hand, many naturally occurring animal tumours are caused by endogenous and exogenous retroviruses, and explants of animal tumours behave in a manner very similar to cells transformed by viruses in vitro (204). Furthermore, though there have been considerably fewer studies of the transformed phenotype resulting from chemically- and radiation-induced transformation, the resulting phenotype seems to be quite similar to that of the virally transformed cells, suggesting that whatever the mechanisms of transformation may be, the end result in terms of phenotype is essentially the same (20, 21, 50, 53, 62).

Thus, in studies where only the behaviour of transformed cells is being studied, not the mechanisms whereby transformation occurred, choice of transforming agent may not be so crucial. The next section describes the transformed phenotype using data drawn mainly from studies of viral transformation, though some data obtained from chemically transformed cells are also included.

### Section B The Transformed Phenotype

Upon transformation, either spontaneous or induced, cells acquire a number of characteristics which usually reflect the generally decreased control of growth and movement, such as loss of contact inhibition of movement, loss of density dependent growth control and loss of anchorage dependence.

#### Contact Inhibition

Contact inhibition was first described by Abercrombie and Heaysman (3). As defined earlier, it is the property by which a normal cell tends to stop moving in a particular direction if it comes into contact with another cell, but is able to move away in other directions. This leads to confluent cultures becoming highly ordered with elongated cells running parallel to each other and forming complex whorls and fan-shaped patterns (ref 204 p405). In confluent

fibroblast cultures, at least, cell movement does not stop necessarily, but a continuous cell streaming occurs (204).

Transformed cultures rarely show much order. Usually the cells grow in a random criss-crossed fashion, with cells piling up on top of each other.

It has been possible to quantitate contact inhibition, and generally it is found that transformed and tumour cells have greatly reduced contact inhibition, which may account for the disorder in transformed cultures (4). Bell (22), however, concluded that the criss-crossed pattern he observed with a culture of polyoma-transformed 3T3 cells was due to the random extension of pseudopodial arms from the cells, not loss of contact inhibition. However, it is probably partly loss of contact inhibition that enables transformed cells to grow and move on top of a monolayer of normal cells. This has enabled identification and selection of newly transformed clones from such foci growing amidst a background of normal cells (ref 115 pp418-420). Efficiency with which dispersed transformed cells are able to grow to form foci on a normal monolayer is a simple quantitative assay for loss of contact inhibition and density independent growth.

#### Density Dependent Inhibition of Growth

Sparse cultures of normal cells grow at a rate only a

little below that of transformed cells, but when a confluent monolayer of cells forms growth often abruptly ceases or slows greatly (158, 197, ref 115 p451, ref 204 pp406 & 409). Transformed cells, on the other hand continue to grow rapidly until their density is 10 to 20 times greater than normal cells, forming cultures which may be several cell layers thick, even in the presence of only very low levels of serum (197, ref 204 pp409-410, ref 115 p451).

It was at one time thought that contact inhibition prevented growth of normal confluent cultures (158, 159, 241, ref 115 p451); contact with cells on all sides inhibited a cell's further growth. However, many nontransformed cell lines may actually grow to quite high density if the supply of serum or growth factors is increased (ref 115 p451). In such dense cultures of normal cells, unlike transformed cultures, the orderly arrangement, which is attributed to contact inhibition, is retained. Stoker and Rubin (241) proposed the term 'density dependent inhibition' to describe this growth control phenomenon, which may be somehow linked to the supply of serum growth factors (ref 115 p451). Folkman and Greenspan (72) proposed that cell shape may be important in determining a normal cell's ability to grow. This was supported by Folkman and Moscona (73) who coated plastic Petri dishes with a nontoxic substance called poly (2-hydroxyethylmethacrylate) (poly(HEMA) ) at a range of concentrations. This had the effect of varying the adhesivity

of the plastic so that on dishes bearing no poly(HEMA) normal cells spread well, whereas with increasing concentrations of poly(HEMA) cells became progressively more round. They found that the rate of DNA synthesis in any sparse culture was inversely proportional to the vertical thickness of each cell. In confluent cultures growing on untreated plastic the cells became more rounded, presumably due to cell crowding, and DNA synthesis slowed considerably, to a level very similar to sparse cells which were rounded by poly(HEMA) treatment.

SV40-transformed 3T3 cells grew equally rapidly on all treated and untreated plates, showing that their growth rate was not regulated with cell shape.

Folkman and Moscona suggested that sensitivity to serum growth factors in normal cells is controlled in some way by cell shape, possibly due to changes in surface area, so that flat cells are able to respond to serum and so grow, whereas rounded cells do not grow because they are less sensitive. Increasing the serum concentration should at least partially overcome this and allow the cells to grow. Cell shape and sensitivity to serum may be uncoupled by transformation and thus allow transformed cells to grow to very high densities regardless of morphology and serum concentration.

#### Anchorage Dependence

Stoker et al. (242) first applied the term 'anchorage



dependence' to describe the property of non-transformed cells whereby they are obliged to attach to a solid support in order to grow, unlike transformed cells which are able to grow freely in suspension. Macpherson and Montagnier (173) developed a method to grow single transformed cells into colonies suspended in semi-solid agar. This technique has proved very useful for isolation of transformed clones from a background of normal cells, and for quantitation of the degree of anchorage independence possessed by different transformed clones.

How cells lose anchorage dependence is not known but it may be explained by the coupling of cell shape to growth control, outlined above. Cells in suspension are spherical and therefore under the theory put forward by Folkman and Greenspan (72) normal cells will be unable to grow, possibly due to insensitivity to serum growth factors. Transformed cells will grow due to uncoupling of cell shape from growth control. A role of cell shape in growth control is supported by the observations that normal cells will grow suspended in agar if glass beads large enough for the cells to attach and flatten out are included in the agar. Silica particles to which the cells can anchor but are too small for them to spread out will not support their growth (72). This would suggest that anchorage per se is not sufficient for cell growth, rather that cell flattening may be.

Clearly these three basic features of normal cell

behaviour are profoundly changed by transformation. These changes involve cell-cell communication, cell morphology and nutrient requirements, all of which are closely related to cell surface phenomena. It is not surprising, therefore, that many of the biochemical changes so far identified in transformed cells involve, either directly or indirectly, the cell surface. It is still not clear, however, which changes may be primary lesions causing the transformed phenotype and which are secondary changes occurring as a result of transformation.

The changes so far identified include rounded cellular morphology and partial dissolution of the cytoskeleton (197, 203, ref 115 pp444-447), altered plasma membrane lipids and glycolipids (ref 189 pp5-6, ref 115 pp436-9), decreased cell surface fibronectin (259, 274, ref 128 pp78-82, ref 189 pp7-8), collagen (ref 128 p85) and glycosaminoglycans (263, ref 189 p5), new cell surface antigens (150, ref 189 pp34-43), increased cell agglutinability (ref 115 p443, ref 189 pp19-34), increased uptake of certain amino acids (188, ref 189 p14) and sugars (ref 115 pp441-3, ref 189 p14), secretion of proteases (ref 115 p439) and other proteins (88, 226) and reduced levels of cyclic AMP (197, ref 115 p447).

Very few transformed clones possess all these characteristics; usually only a few are expressed. Some properties may not be expressed immediately following transformation

but may manifest themselves some time later. In vivo tumourigenicity is the ultimate test of a transformed clone. No single phenotypic marker for transformation correlates completely with in vivo tumourigenicity, but some, such as growth in agar, are better indicators than others (75, 139). This will be discussed in detail later.

Normal cells under certain conditions can actually be made to temporarily mimic the transformed state. For instance, cells in mitosis or when mildly treated with protease are rounded, have lost the microfilament system and cell surface fibronectin, and are more agglutinable (ref 189 p12).

#### Morphology and Cytoskeleton

Normal fibroblasts usually have a well-spread flattened morphology and are firmly adherent to the substratum. Cell shape, adhesion and motility are largely controlled by the cytoskeleton (39, 149, 156), which consists of microtubules, intermediate filaments and microfilaments.

Microtubules, consisting principally of tubulin, and intermediate filaments, consisting of desmin, form two separate lattices throughout the cytoplasm (130, 193, 266, 267). Microfilaments, containing mainly actin, exist in two arrangements (149):

a) as single filaments dispersed throughout the cytoplasm, in the perinuclear region and associated with the leading ruffling edge of moving cells, and b) in well-spread cells,

as bundles (sometimes called stress fibres) running in straight parallel arrays along the lower surface of the plasma membrane ( 87, 155, 156, 203).

Upon transformation the cells usually become smaller, rounded and less adherent to the substrate. Some observations have suggested that the microtubule system may break down upon transformation (37, ref 115 pp444-7), but in the majority of cases at least, it now seems that the microtubules remain intact (60, 192). Similarly, the intermediate filaments remain essentially unchanged (130). The microfilaments, however, particularly the stress fibres in most transformed lines are either lost or at least greatly reduced. The total amount of cellular actin may or may not be reduced (70, 215, ref 128 p88), but is probably present in the cytoplasm in the monomeric form instead of the polymerised state (ref 128 p88).

Loss of the stress fibres probably at least partially explains the rounded morphology and weaker adhesion (ref 128 p122).

#### Cell Surface Components

Alterations in synthesis of various plasma membrane lipids, glycolipids, glycosaminoglycans and phospholipids have been reported but much of the data are conflicting and few generalisations can be drawn (115, 189, 262).

Some experimental evidence has suggested that the

fluidity of the plasma membrane is increased after transformation, but the weight of evidence which has accumulated against this possibility now makes it seem unlikely (ref 115 p444).

Normal cells secrete onto their outer surfaces a matrix which aids adhesion to the substrate (259, ref 128 p70). This matrix, or glycocalyx, is known to consist of at least three principal components; fibronectin, collagen and glycosaminoglycans (259), and seems to be involved in adhesion of cells to the substrate.

In transformed cells this glycocalyx is greatly reduced. The glycosaminoglycans are still produced and secreted in large quantities but they are not retained on the cell surface (ref 259 pl6). Synthesis of collagen and fibronectin is reduced, and in addition, fibronectin which is synthesised and secreted is not retained on the cell surface as efficiently as on normal cells (191, ref 259 pl4 & 17).

Fibronectin, LETS (Large External Transformation-Sensitive protein) or CSP (Cell Surface Protein) as it may be known (amongst other titles), is a large glycoprotein with subunit molecular weight of  $2.2$  to  $2.5 \times 10^5$  daltons (259, 274, 275, 278).

Primary explants of both epithelial and fibroblastic cells synthesise and secrete onto their surfaces large amounts of fibronectin while established cell lines produce much lower amounts and transformed cells may have little,

if any, fibronectin. Its absence was thought to correlate very closely with in vivo tumourigenicity (259, 274), but several tumourigenic transformed lines have recently been found to possess levels of fibronectin similar to the parental non-transformed line (274).

Antisera raised to fibronectin of one species cross-reacts with fibronectin of a range of species, suggesting a conserved structure (279, 286). Immunofluorescence using such antisera reveals several different patterns on cells of primary explants (177, 274). Confluent cultures possess a complex intercellular filamentous matrix running across the upper surfaces of the cells. Subconfluent cultures show mainly 'stitches' of fibronectin between adjacent cells, with very little matrix running across the cells. Extraction with NP40 allows the antisera to stain fibronectin beneath the cells which forms a filamentous network across each cell, with some intercellular connections (131, 177).

Addition of fibronectin to cultures of transformed cells often results in their adopting a more normal phenotype-flattened, more orderly alignment and containing micro-filament bundles (6, 274, 277), and also increased cell motility (7). Again, fibronectin from one species is active in a range of other species of cells (6, 277). There is no effect, however, on growth rate, saturation density or nutrient transport (6, 274, 277).

Other data suggest considerable links between fibronectin

and microfilaments. In normal cells immunofluorescent staining patterns for actin and fibronectin reveal contiguous fibres with microfilaments inside the cells and fibronectin outside (131), treatment with proteases or cytochalasin B disaggregates both microfilaments and fibronectin fibres, and mitotic cells too lack both structures (128, 189, 259).

For interactions between the microfilaments and fibronectin fibres to occur there must be a third component in the plasma membrane since neither actin nor fibronectin have yet been identified as intramembranous proteins. Evidence for such a component is increasing, though its identity is still a mystery (128, 131, 230). Thus it seems probable that microfilaments and fibronectin together play a major role in facilitating cellular adhesion and controlling cell morphology, but apparently having little effect on control of growth.

Disruption of this microfilament-fibronectin complex in transformation would clearly result in considerable changes in cellular behaviour.

#### Increased Agglutinability

Transformation is usually associated with increased agglutinability of suspended cells by lectins such as concanavalin A (con A) and wheat germ agglutinin (WGA) (51, 195, ref 115 p443, ref 189 ppl8-34). Normal cells during

mitosis or when subjected to mild protease treatment mimic this increased agglutinability (ref 115 p443, ref 189 p24).

Transformed cells do not bind more lectin, rather cross-linking of bound lectin molecules with each other on the same and adjacent cells is somehow facilitated (51, 195, ref 189 p22). This facilitation is probably achieved by improved mobility of lectin-bound receptors within the plasma membrane. Control of mobility of surface receptors is thought to be by a structure called the Surface Modulating Assembly, consisting of microtubules, microfilaments and possibly fibronectin (65, 276, ref 128 pp95-99, ref 189 pp26, 29-31). Partial disruption of the SMA by transformation through loss of microfilaments and fibronectin would free the receptors from limitations on their mobility. No direct experiments have proved the existence of the SMA, but experiments with drugs which disaggregate microtubules and microfilaments resulting in increased agglutinability argue strongly in its favour (65, 66, 178, 179, ref 189 p30).

A number of reports have shown that raised agglutinability with con A, but not WGA, correlated quite well with in vivo tumourigenicity (ref 189 p22). Recently a 100000 dalton cell surface glycoprotein was found to bind proportionally more con A, but less WGA, following transformation than before (273). A range of transformed cells which showed this alteration were also tumourigenic in vivo, whereas those without it were not. WGA is toxic for transformed cells and



so was used for selecting normal revertants. Such revertants showed decreased con A binding to this 100K glycoprotein, as well as being non-tumourigenic (273).

#### Induction of Cell Surface Antigens

Both chemically transformed cells and some human tumour cells express on their surfaces common embryonic antigens and tumour-specific transplantation antigens which vary with cell type and carcinogen (ref 115 p440, ref 189 pp34-36).

Retrovirus-transformed cells also express cell surface antigens (CSA) which appear to be mainly virus-coded, consisting of, or at least associated with, precursor polypeptides of the gag and env structural genes (150, 235).

These CSA's of course make such cells antigenically distinguishable from normal cells and will thus elicit an immune response in vivo which will limit the spread of tumours caused by these cells (150).

#### Increased Nutrient Uptake

The uptake of glucose and its nonmetabolisable analogues 2-deoxyglucose and 3-O-methylglucose as well as mannose, galactose and glucosamine are all stimulated following transformation, whereas the uptake of other carbohydrates is unaffected (ref 115 p442, ref 189 p14). Similarly the amino acids glutamine, arginine glutamic acid and alpha aminoisobutyric acid are reported to be transported more

readily (ref 189 pl4), though a recent report by Nakamura and Weber (188) found no differences in amino acid uptake between normal and chick embryo fibroblasts transformed by Rous Sarcoma Virus (RSV) unless they were starved of amino acids first.

The reduced requirement for serum by transformed cells may be attributable to increased uptake of nutrients from the medium, or perhaps to increased sensitivity to growth factors in the serum.

### Secretion

Transformed cells and tumours both in vivo and in vitro secrete proteases which, it has been proposed, may be partly responsible for the transformed phenotype, for instance by cleaving away cell surface fibronectin (ref 115 p439, ref 128 pp91-92, ref 259 pl1).

One such protease is plasminogen activator which converts plasminogen (normally found in the serum in culture media) to plasmin, to which fibronectin is very sensitive (ref 128 p91). This may be one cause of the loss of fibronectin from cell surfaces, but the evidence so far suggests that it is at best only a minor cause (ref 128 pp91-92).

Senger et al. (226) have recently identified two classes of transformation-specific proteins, both with molecular weights of about 60000 daltons, and one of which is phosphorylated. These proteins are secreted from cells of several

different mammalian species transformed by a range of type C viruses. However, it is still not clear just how general these proteins are throughout the whole range of transformed and tumour cells, nor is their role in transformation understood.

#### Cyclic AMP

Cyclic adenosine-3',5'-monophosphate (cAMP) has been implicated many times in the control of the growth of cells (197, ref 115 p447). Several normal cell types, e.g. Swiss 3T3 and NRK, when growing rapidly have quite low levels of cAMP, but as confluence is reached the amount present rises by about two fold or more (197). In transformants of these cells cAMP levels always remain low (197). There are, however, several normal cell lines which do not show this fluctuation, such as BALB/c 3T3 and chick embryo fibroblasts (197), so the significance of cAMP is questionable.

The best evidence for a role of cAMP in control of cell behaviour comes from experiments on the effects of treatment of transformed cells with cAMP or dibutyryl cAMP. Transformed cells treated with dibutyryl cAMP became flatter, more adherent and arranged in orderly parallel arrays, cytoskeleton is restored, growth is slower and the cells are less agglutinable (197, 205, ref 115 p448). This effect can be potentiated by pretreatment with testosterone (205).

The physiological significance of results obtained with dibutyryl cAMP is in doubt since butyric acid has similar effects on morphology (8, 83, 85, 171, 271), though Storrie et al. (243) found that the effects were not identical. Butyric acid-treated transformed cells become flatter, have a restored cytoskeleton, raised intracellular cAMP levels and grow more slowly (as do normal cells in the presence of butyric acid), but density dependent growth control is not restored (8). Altenberg et al. (8) found that butyric acid did not restore adherens junctions between cells, a type of junction which is common between normal cells. They proposed that this may be why growth control was not restored.

#### Correlation of In Vitro Transformation with In Vivo Malignancy

Many of the observations regarding behaviour of transformed cells often apply well to cells of naturally occurring tumours explanted into tissue culture. They are small, rounded, generally lack a microfilament system and cell surface fibronectin, grow in agar, grow to high densities on a plastic substrate and are highly agglutinable. Thus it would seem that the in vitro transformed phenotype is a realistic equivalent to in vivo tumours. It is probable that certain of the in vitro properties correspond with specific in vivo features of tumours. Thus, loss of contact

inhibition of movement in vitro may correspond to invasive-ness in vivo (4, ref 204 pp405-6), loss of density dependent growth control to uncontrolled irregular growth in vivo (ref 189 p22, ref 204 p410) and anchorage independence to ability to metastasise (50).

Similarly the expression of a number of in vitro phenotypic characteristics of transformed cells have been shown to correspond quite well with their ability to form tumours in vivo. Such characteristics include growth in agar (50, 75, 139), loss of cell surface fibronectin (274), growth to high saturation densities (1, ref 189 p22, ref 204 p410) and raised agglutinability by con A (272, 273, ref 189 p22). None of these correlates fully with in vivo tumourigenicity (23, 44, 137), but the combination of the four is generally sufficient to identify a tumourigenic clone.

Growth in agar is probably the most widely used method, reportedly correlating very closely with in vivo tumourigenicity (50, 53, 75, 139, 229). Loss of cell surface fibronectin was thought to correlate completely with in vivo tumourigenicity, but a number of exceptions have been discovered (274). It is now thought to correlate more closely with the ability to metastasise in vivo or in vitro growth in agar (274). Growth to high saturation density was probably one of the first phenotypes to be equated with in vivo tumourigenicity (ref 189 p22, ref 204 p410). Many exceptions have been found (ref 204 p410). though it is still useful as a rough guide.

Con A agglutinability generally parallels loss of growth control (ref 189 p22), so its consequent correlation with tumourigenicity is not surprising. This may also relate to the 100000 dalton marker for malignancy mentioned earlier (273). Agglutination by other lectins, such as soybean and wheat germ agglutinins, does not correlate with tumourigenicity.

#### In Vivo Tumourigenicity

The ultimate test to equate in vitro transformation with in vivo carcinogenesis is to inoculate animals with transformed cells and watch for the growth of tumours.

The need for histocompatibility of cells and hosts restricts in vitro work to use of cells explanted from inbred mice. Transformed cells can then be inoculated into syngeneic hosts. This limitation has been partly overcome by the introduction of nude mice which are athymic and so are unable to mount T cell-mediated immunity (196, ref 204 p413). These mice are able to take allogeneic grafts, including those from other species (207). However, these mice can mount humoral immunity and Natural Killer (NK) cell activity which could destroy allogeneic grafts.

Due to the lack of T lymphocytes these mice are very susceptible to infections which may prove lethal, and so are very difficult to handle. It is at least partly for this reason that the majority of studies are still conducted using inbred syngeneic cells and animals.

## Section C RNA Tumour Viruses -

### Tools for Transformation

Since they transform many cell types with relative ease, RNA tumour viruses, or retroviruses, are the most widely used transforming agents. They have also been used in these studies.

The major class of retroviruses is the C-type viruses, which itself consists of two principal divisions: the sarcoma and leukaemia viruses. The former transform cells in vitro, whereas the leukaemia viruses can be divided into acute leukaemia viruses (ALV) which transform haematopoietic cells in vitro (and sometimes fibroblasts too), and lymphatic leukaemia viruses (LLV) which do not transform cells in vitro but do cause lymphomas in vivo, though only after a long latent period (ref 115 pp402-8).

There is a further division into replication competent and replication defective viruses. In avian systems the sarcoma viruses and LLVs are competent, though there are some Avian Sarcoma virus (ASV) mutants which are replication defective (ref 115 pp402-8). In mammals all sarcoma viruses are replication defective, while the LLVs are competent. ALVs of both avian and mammalian origin are defective. Replication of defective viruses requires the presence of 'helper' LLVs, so that all mammalian sarcoma virus and ALV stocks contain LLV.

By infecting cell cultures with virus at high dilution

double infection with sarcoma and leukaemia viruses will be rare, so the majority of cells will be infected with just a sarcoma or a leukaemia virus. Transformed clones can then be isolated by focus formation or by culturing in agar. These will be infected with a sarcoma virus only, and so will show no viral replication. Such transformed clones which produce no virus are called 'non-producers'. The sarcoma virus in these non-producers can be 'rescued' by superinfection with a LLV, which will provide all the proteins necessary for virus production. The rescued sarcoma virus will be antigenically identical to the helper virus.

Initial infection of cells with sarcoma or leukaemia viruses follows essentially the same path. The viral RNA is transcribed by viral reverse transcriptase into DNA, which is circularised and then integrated into the host cellular genome (52, ref 115 pp410-414). Integration and the subsequent initiation of transcription of the integrated provirus requires a cellular process, possibly some event late in G2 or mitosis (ref 115 pp414-5), the reason for which is obscure.

Replication competent viruses synthesise the full complement of structural proteins from the provirus, whereas the defective viruses are able to make only a few (235), or in some cases none at all (235, 282).



Relationship between Mammalian Sarcoma and Lymphatic  
Leukaemia Viruses

The most closely studied mammalian viruses are those of the murine system from which a number of LLVs have been isolated. Several strains of sarcoma viruses have been experimentally derived from these LLVs by serial passage through animals. Both the Kirsten and Harvey sarcoma viruses were derived experimentally by passage of LLV through rats, the former being derived from mouse erythroblastosis virus (MEV), which is now usually called Kirsten leukaemia virus (KiLV) (ref 282 p256). Harvey sarcoma virus was derived from Moloney leukaemia virus (MoLV) (ref 282 p256), which was also passed through mice to give rise to Moloney sarcoma virus (MoSV).

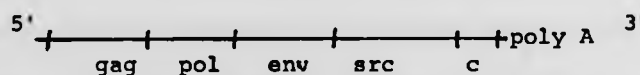
The new viruses arose due to recombination of the leukaemia virus with the cellular genome, thus losing viral structural genes and gaining cellular genes (227, 228, 235, ref 115 pp426-8, ref 282 pp255-257). This is the cause of the defective nature of sarcoma viruses.

The proportion of leukaemia virus genome remaining in the different sarcoma viruses varies greatly. For instance, only 15% of the Kirsten sarcoma virus (KiSV) genome is homologous with Kirsten leukaemia virus (KiLV), whereas 75% of the MoSV genome hybridises with MoLV (ref 282 p255). The sarcoma virus-specific sequences are of cellular origin; in the case of KiSV and Harvey sarcoma virus (HaSV) much of it may be from a rat endogenous virus (235, 282).

### Structure of the Genome

The genome of all C-type viruses is single-stranded RNA, present in the virion in diploid form, each copy having a sedimentation value of about 35s in the fully competent form, but only 30s in the replication defective mammalian sarcoma viruses (52, 282, ref 115 pp421-6).

Probably more is known about Rous sarcoma virus (RSV) than any other C-type virus. It is a fully competent, transforming virus of chickens. Its genome consists of four genes (52, ref 115 pp421-6), designated thus -



The gene gag codes for the viral core proteins, pol for the virion reverse transcriptase, env for the envelope glycoprotein(s) and src codes for the cell transformation protein(s). Towards the 3' end is a sequence designated 'c' which is common to all type C viruses, with no known function. Right at either end of the genome are short homologous sequences which may be involved in integration of the DNA transcript into the host genome (ref 52 p5).

The lymphatic leukaemia viruses, both avian and mammalian, possess the three structural genes, but not src.

Murine sarcoma viruses usually synthesise some of the gag viral proteins in nonproducer transformed cells, most often those nearest the 5' end, indicating that this region

of the leukaemia virus is retained (150, 235). However, both KiSV and HaSV express no viral genes because the leukaemia sequences at the 5' end are too short to code for any proteins (ref 282, p258). In these two viruses most of the leukaemia-related sequences reside in the non-translated sequences at the 3' end of the genome (227, 282).

#### Src Gene and Transformation by RSV

DNA complementary to the RSV src gene (cDNAsarc) hybridises to approximately 10 - 15% of the RSV genome (ref 115 p427). This cDNAsarc not only hybridises to the integrated proviral sequences in infected cells, but also to the DNA of uninfected cells (154, ref 115 p427). The viral src and cellular sarc sequences in chicken cells are not completely identical. Hybridisation also occurs between cDNAsarc and cellular sequences of other avian species (ref 115 p427), though the degree of mismatching increases as the evolutionary distance between chickens and the species being studied increases. No hybridisation occurs with LLV of avian or mammalian origin, nor with any mammalian sarcoma viruses.

These data suggest that the viral src of RSV and cellular sarc of a range of avian species had a common ancestral origin from which there has since been divergent evolution.

The gene product of src has been identified as a

phosphoprotein of molecular weight 60000 daltons, designated pp60<sup>src</sup> (42, 154, 225, 228), possessing protein kinase activity. The pp60<sup>src</sup> has to be constantly present in order to maintain transformation (206, ref 115 p423). Mutants of RSV temperature sensitive for transformation show rapid transition between the transformed and normal state on shifting between the permissive (36°) and nonpermissive (41°) temperatures. Some changes occur within 30 minutes of temperature shift, and transition is completed within 24 hours (206, ref 115 p432). One of the earliest changes to be seen on shifting to the permissive temperature is the dissolution of the cytoskeleton (66, 179, 197, 262), so it has been proposed that at least one of pp60<sup>src</sup>'s main actions may be a direct effect on the cytoskeleton, causing the microfilament system to breakdown (129, 179).

Recently it has been found that pp60<sup>src</sup>'s protein kinase activity is independent of cyclic AMP (154), and instead of phosphorylating serine or threonine residues, which are the normal substrates, it phosphorylates tyrosine (127, 154, 225). Levels of phosphotyrosine in RSV transformed cells are ten fold higher than the levels found in uninfected cells (129, 225), autophosphorylation and phosphorylation of two cellular proteins at 34K and 50K daltons apparently being the main targets (127, 154, 225).

In uninfected cells a phosphoprotein with a molecular weight of 60000 daltons has been detected (154), which

crossreacts with antisera to pp60<sup>src</sup>, and which phosphorylates tyrosine residues. The levels of this protein in normal cells are 50-60 times lower than the levels of pp60<sup>src</sup> in transformed cells. This protein is probably the product of cellular src genes, but its function is obscure.

#### Transforming Genes of Murine Sarcoma Viruses

Recently a phosphoprotein of molecular weight 21000 daltons, designated p21, has been identified in cells transformed by KiSV and HaSV. Antiserum raised in rats bearing tumours induced by syngeneic inoculation of HaSV-transformed normal rat kidney (NRK) cells precipitated p21 from both KiSV and HaSV-transformed cells and p21 translated in vitro from KiSV mRNA (227). KiSV mutants, temperature sensitive for transformation, produce thermolabile p21, suggesting that this protein, analogous to pp60<sup>src</sup> of RSV, may be the transforming protein for at least these two murine sarcoma viruses (228).

Neither Kirsten nor Moloney leukaemia viruses synthesise this protein, so it is probable that the rat cellular sequences in the sarcoma viruses code for it. This possibility is strengthened by the antigenic identity of p21 from KiSV and HaSV transformed cells. Whether these sequences have a role in rat cells is not known.

It would seem that the transforming genes so far

identified, src for RSV and p21 for KiSV and HaSV, are all derived from cellular sequences. It is still not known what function, if any, these cellular genes might normally have, or why they do not cause transformation in uninfected cells. It was noted earlier that the levels of viral pp60<sup>src</sup> in transformed cells are 50-60 times higher than the levels of the sarc protein in normal cells, so transformation may be the result of excessive quantities of a protein normally present in very small amounts. It has been suggested that the sarc protein has an important role in regulating growth, and hence the conservation of its gene sequence throughout avian species. Transformation by pp60<sup>src</sup> could be due to its much higher concentration upsetting this normal control, by perhaps overactivating a normal process (154). One possible candidate is the control of binding of Epidermal Growth Factor (EGF). Binding of EGF to the cell surface is accompanied by phosphorylation of tyrosine residues of a cell surface protein, possibly the EGF receptor (154).

Even if phosphorylation of tyrosine residues is important in transformation by RSV, it does not appear to be universal. Mouse cells transformed by KiSV, MoSV or SV40, chick embryo cells infected with avian myelocytomatosis virus MC29, rat or hamster cells transformed by polyoma virus, and at least three chemically transformed lines show no increase in phosphotyrosine levels (225).

PART 2INTERFERON'S POTENTIAL AS AN ANTITUMOUR AGENTSection A Historical Perspective

For a considerable period before interferon was discovered it was known that virus infection of a tissue could prevent subsequent infection with a second virus. This was first observed in 1935 by Hoskins (ref 236 p2) who termed it 'viral interference'

It was not until 1957 that Isaacs and Lindenmann clearly demonstrated a diffusible agent, produced by virus-infected cells which could interfere with virus replication (132, 133). They took chick chorioallantoic membranes and infected them in vitro with heat-inactivated influenza virus. After 20 hours they removed the supernatant fluid and added it to uninfected membranes. These were then challenged with live influenza virus, which failed to replicate. They concluded that the inactivated virus had stimulated the infected cells to synthesise and secrete a substance which rendered the uninfected membranes resistant to live virus. This substance they called the 'interferon'.

Interferon was found to be a glycoprotein, stable at pH2, though an acid labile form was later found, whose production could be stimulated by a range of substances other than viruses. It would protect a range of tissues against infection by many different viruses, but interferon from any

one species was active only in cells of that species - its activity was said to be species-specific.. This restriction is now known not to be rigid and the term 'restricted host range' is more appropriate. This will be discussed in more detail later.

For a number of years interferon was considered to be a purely antiviral substance with no effects on the host cells' metabolism, despite effects on cell morphology reported by Gresser in 1961 (89), and inhibition of DNA synthesis reported by Paucker et al. in 1962 (198). Such effects were generally considered to be due to impurities in the interferon preparations (19, 160, 182, ref 236 p238).

However, it was found that the growth of tumours in vivo could be inhibited, initially virally induced tumours where the inhibition was attributed to interferon's antiviral activity (94), but subsequently it was shown that growth of transplantable tumours which were not virus induced could also be inhibited (91, 99, 102). These findings paralleled further reports that in vitro cell growth was slowed by interferon (100, 101, 172).

The mechanisms by which interferon inhibits tumour growth in vivo and cell growth in vitro are still unknown, but the antiviral mechanism is now quite well understood (11, 13, 15, 17, 113, 123, 142a, 208, 218, 270). These mechanisms will be discussed in more detail later.

Interferon has been used on a number of occasions to



treat cancer sufferers but always only very small numbers of patients have been treated in each test and none have been double-blind controlled. The most frequently quoted trial has been treatment of a rare bone tumour, osteosarcoma, which involved some 30 patients in Sweden (94, ref 236 pp320-1). Though beneficial effects were seen, the trial was not double-blind controlled.

One of the major stumbling blocks to clinical trials is the shortage of supply of interferon. Present doses used are up to several million units per week. The bulk of the world's supply of human interferon has been produced from buffy coats by the Finnish Red Cross, and more recently from lymphoblastoid cells by Wellcome Research Laboratories, but these are only sufficient for a limited number of small trials.

Increased supplies may eventually be available from constitutive production of large quantities of human interferon following manipulation of the human interferon genes into Escherichia coli (187).

## Section B    Properties of Interferon

### Types of Interferon

The concept of restricted host range of activity implies that interferons of different species have different structures, so that the term 'interferon' represents a whole range of closely related glycoproteins.

The most closely studied interferons are those of mice and humans. There are at least three major classes of human interferon, called under a new classification system, alpha, beta and gamma. Alpha and beta are the classic virally induced pH 2 stable leukocyte and fibroblast interferons respectively, formerly called type 1, while gamma corresponds to the pH 2 labile immune or type 2 interferon induced in lymphocytes and macrophages by antigens and mitogens (9). Purified alpha interferon appears on polyacrylamide gels as two bands at 15-18000 daltons and 21-22000 daltons (36, ref 236, ppl65 & 174). Removal of the carbohydrate moiety by periodate treatment or allowing cellular synthesis to proceed in the presence of tunicamycin converts all alpha interferon to the 15000 dalton form (36, 239, ref 236 pp 167 & 180), suggesting that if there are different primary amino acid sequences, they are all closely related.

Purified beta interferon appears on polyacrylamide gels as just one band at 19-20000 daltons. Deglycosylation of this interferon has little effect on its mobility (24, 145, 249, ref 236 pl81).

Using highly purified material the amino terminal sequences of human beta (147), lymphoblastoid (285), and mouse Ehrlich Ascites (245) interferons have been determined, and the entire sequences of human alpha and beta interferons have been deduced by sequencing the cDNAs used to clone the

genes into E. coli (61, 250). The results show that alpha and beta interferons have different sequences, but the genes are closely related, and presumably have diverged from a common ancestor. Lymphoblastoid interferon which is produced by established human lymphoma lines appears to consist principally of alpha interferon with a few amino acid changes (250, 284).

Interferon from mouse Ehrlich Ascites cells exists in three forms, A, B and C, with molecular weights at 35-40,000 daltons, 26-33000 daltons and 20000 daltons respectively (45, 245). The partial sequence data have shown that A and B probably have an identical sequence which is closely related to human beta interferon. C has a sequence which is related to human alpha interferon. Therefore A and B are classed as beta and C as alpha interferons.

Interferon from mouse L cells consists of just two forms, at 35-40000 daltons and 22-28000 daltons. Deglycosylation converts both to one smaller form at 18000 daltons, and though it has yet to be sequenced, it is thought to be principally beta type interferon (58, 134), though Yamamoto and Kawade (280) and Trapman (257) recently found the two forms to be antigenically distinct.

Gamma interferons consist of all the type 2 interferons that have so far been identified in different species. Mouse gamma IFN was first identified in mice that developed antiviral resistance following injection of certain antigens.

No alpha or beta interferons could be detected in the serum of these animals, but eventually an acid-labile interferon-like substance was identified (ref 236 ppl45 & 8). Mouse and human gamma interferons are heterogeneous glycoproteins stable at pHs down to about pH4 (ref 236 ppl45 & 8).

All three interferons, alpha, beta and gamma have both antiviral and anticellular activities, but the facts that gamma is not induced by viruses and may have more potent anticellular activities, and that human beta in vivo is more quickly removed from serum than human alpha, suggest that the different interferons may have rather different roles in the body (ref 236 pl82).

#### Some Characteristics

Restricted host range Interferons do not show strict species specificity (ref 236 ppl35-145). Both human alpha and beta interferons are active on monkey and rabbit cells (ref 236 ppl36-7) both in vivo and in vitro, but only alpha IFN is active on bovine and cat cells (ref 236 pp 136-7). Similarly mouse IFN shows some activity on rat cells, but is actually more active on guinea pig cells than on mouse cells (ref 236 pl42).

The reverse is not necessarily true, however - monkey IFN is active on human cells, but rabbit IFN is not (ref 236 pl38).

Acid stability As already described, alpha and beta interferons are stable at pH 2, which has proved very useful in an initial purification step, but gamma interferon is stable at pH 4 but not in more acid solutions (ref 236 p145).

Stability to reducing agents and detergents Interferons are generally labile to boiling, repeated freezing and thawing, and physical agitation. Sodium dodecyl sulphate (SDS) protects interferon against such treatments. Mercaptoethanol and urea alone or with SDS have varying effects on different interferons. For instance human alpha interferon is protected by SDS or mercaptoethanol alone, but is only partially protected by the two together. The response of human beta interferon, however, is completely the reverse (ref 236 p175).

## Section C    Production and Purification

### Inducers

A whole array of substances induce interferon production in vitro and/or in vivo. Viruses of every major class induce interferon, including many inactivated viruses (ref 236 p34). Synthetic double-stranded RNAs may also be potent inducers, particularly poly rI.poly rC (ref 236 pp46 & 49).

Various bacterial products, such as statolon from Penicillium stoloniferum induce interferon, because of the double-stranded RNA viruses they contain. Double-stranded RNA appears to be the active component in many inducers, but there are many others which are structurally unrelated, such as bacterial endotoxins, radioprotective chemicals, synthetic polyanions, e.g. pyran copolymer and low molecular weight substances such as tilorone hydrochloride. Many of these only induce in vivo, and no common molecular structure can be found which may suggest a common induction mechanism (ref 236 pp52-54).

#### Yields

Cell type and inducer must be closely matched in order to achieve maximum yields since different inducers and cell types vary in their potency and sensitivity towards each other. As a result routine production of IFN is generally conducted in a very limited number of ways, such as mouse L cells with Newcastle Disease Virus (NDV) or human diploid fibroblasts with poly rI.poly rC.

Yields can be greatly increased in some inducer-cell type systems by pretreating the cells with small amounts of interferon before induction, a process called priming (ref 236 p233). Superinduction is often used coupled to poly rI.poly rC induction. This involves the application of cycloheximide and later actinomycin D to induced cells.

This treatment increases the maximum yield, and duration of production of interferon (ref 236 pp97-100).

#### Purification

Purification to homogeneity has only recently been achieved for mouse and human alpha and beta interferons (24, 45, 58, 145, 147, 216, 224, 249, 284). The procedures usually involved concentrating large volumes of crude interferon to a manageable size and then purification was achieved by various combinations of gel filtration, affinity chromatography and polyacrylamide gel electrophoresis. Most of the multistep procedures developed give yields of 1-10% of the crude starting material (24, 134, 141, 145, 147, 249), whereas some two-step methods may give 50-90% yields. In this respect one of the most successful procedures for purifying mouse interferon is that described by de Maeyer-Guignard et al. (58). Crude interferon from mouse C243 cells was purified by affinity chromatography on poly U-Sepharose followed by chromatography on anti-interferon antisera coupled to an agarose column. In this way 90% of the starting material was recovered in two fractions. The first fraction carried a few contaminants, but the second was pure as judged by polyacrylamide gel electrophoresis. The specific activity of the pure material was  $2.3 \times 10^9$  international units per mg protein.

## Section D    Cellular Actions of Interferon

### Antitumour Activities in Vivo

Interferon was first shown to have an antitumour effect due to its ability to prevent viral induction of tumours in hamsters and mice (94). The interferon had to be administered prophylactically presumably to induce an antiviral state in the target tissues. Interferon treatment after virus inoculation was generally ineffective, except with RNA tumour virus inoculation, the most closely studied of which was Friend virus inoculation (95, 96, 97, 98, 220). This virus normally infects the spleen and causes rapid growth and multiplication of the spleen cells, the virus itself undergoing multiple rounds of replication, the progeny virions infecting previously uninfected cells. Repeated intraperitoneal interferon injection following infection could successfully block splenomegaly (95, 96), even if treatment was not started until several days after virus inoculation (96, 97). Virus titres and the numbers and size of foci of Friend cells in spleens of IFN-treated mice were greatly reduced, suggesting that the replication and spread of the virus was prevented, although Gresser and his colleagues did also suggest that foci might be reduced in size due to an inhibition of cell growth as well as spread of virus (96, 98).

Gresser and his colleagues subsequently confirmed that interferon could block the growth of tumours by inhibiting



cell growth (91, 99, 102). They found that BALB/c, DBA/2 and C57BL/6 mice could be protected against inoculation of a range of tumour cell types, none of which were virus producers, provided interferon treatment was continued for several weeks after tumour cell inoculation. Prophylactic treatment was ineffective. Interferon was most effective in BALB/c mice inoculated with  $10^3$  Ehrlich Ascites (EA) cells, where almost all interferon-treated mice survived beyond 60 days post-inoculation, whereas no control mice lived more than 30 days. Furthermore an inoculum of one to ten EA cells constituted one LD50 in control BALB/c mice, but  $10^4$ - $10^5$  EA cells were required for one LD50 for interferon-treated mice (91).

Injection of interferon inducers could be just as effective as interferon itself at preventing splenomegaly in Friend disease and tumour growth following RC19 cell or L1210 cell inoculation, presumably by inducing endogenous interferon (90, 268, 269, 283). Levy *et al.* (161) found that the interferon inducer poly(rI)poly(rC) was able to delay the growth of several tumours in mice if administered repeatedly after cell inoculation. A reticulum cell sarcoma and human adenovirus 12 transformed mouse cells were the most sensitive of the transformed cells tested. Even if these tumours were allowed to grow to a palpable size before poly(rI).poly<sup>(rC)</sup> treatment commenced tumour regression still occurred. The sarcoma regressed completely and did not

return even after treatment ceased, whereas the adenovirus tumour regressed only temporarily despite continued poly (rI) poly(rC) treatment.

It was generally found that interferon was most effective if injected by the same route as tumour cell inoculation, and intravenous injection was generally quite ineffective (95, 98). In one instance intravenous injection was effective despite subcutaneous inoculation of cells. Inoculation of Lewis lung carcinoma cells subcutaneously into mice, followed by repeated intravenous interferon injections, commencing 24 hours or 6 days later, inhibited growth of both the primary tumour and pulmonary metastases (92). Both the size of the tumours and the number of tumour-bearing mice were reduced. This result is especially significant since this tumour is generally resistant to most anti-tumour drugs.

#### Spontaneous Tumours

95% of AKR mice in their sixth month develop lymphomas due to the activation of endogenous Gross virus. Treatment of AKR mice with interferon for one year from birth extended the survival time of the mice and decreased lymphoma incidence to 65% (106). Presumably this effect is due at least partly to interferon's antiviral activity.

### Inhibition of Growth of Normal Cells in Vivo

Regeneration of liver following partial hepatectomy in mice (74, 135) and growth of allogeneic lymphocytes or syngeneic bone marrow cells injected into irradiated DBA/2 mice (46) were all inhibited by interferon treatment. Large doses of interferon ( $8 \times 10^5$  units) injected daily into newborn mice caused extensive liver damage which led to death (109). If treatment was stopped after 6 to 9 days the liver repaired itself and the animal appeared healthy, but in succeeding months developed glomerulonephritis and died (94, 110). This could of course have major implications for interferon's clinical application to children.

### Clinical Trials

The discovery of interferon's antitumour activity raised the possibility of interferon's use in cancer treatment. Numerous small tests have been conducted, but all with very small numbers of patients who usually already had a heavy tumour load.

Strander and his colleagues have been conducting a trial since 1971 on interferon's efficacy against osteosarcoma, a rare malignant bone tumour which afflicts mainly young people. With conventional treatment 70% of patients developed metastases and 65% died within  $2\frac{1}{2}$  years of diagnosis. Twenty-eight newly diagnosed patients, who were free of metastases were given daily injections of  $3 \times 10^6$

units of alpha interferon, coupled with excision or X-irradiation of the primary tumour, followed by three interferon injections per week for 17 months. After 2½ years 36% of patients had developed metastases and 28% had died (94). Although these results are encouraging, this trial has been severely criticised because it was not double-blind controlled, the control group that was used consisted of patients receiving treatment at hospitals scattered over Sweden, whereas all members of the interferon group have been treated at the Karolinska Institute in Stockholm, and the numbers of patients involved are so small that the results are not statistically significant.

#### Mechanisms of the Antitumour Activity

It is still not clear how interferon exerts its anti-tumour effect, but there is evidence to suggest that a direct inhibitory effect on the growth of tumour cells, or a stimulation of the host's immune systems, or a combination of the two may be involved.

The observation that interferon is generally most effective when administered via the same route as the tumour cell inoculum suggests that close contact between interferon and tumour cells is required for optimal activity, arguing for a direct effect on their behaviour. When Gresser *et al.* (103) incubated mouse L1210 cells with interferon *in vitro* they found that these cells were less able to grow in agar

in the absence of interferon and were less tumourigenic when injected into animals. Gresser and Bourali (91) further suggested that interferon's inhibition of Ehrlich Ascites tumour formation in BALB/c mice was due to a direct effect on cell growth since such cells grew much more slowly in the peritoneal cavity of IFN-treated than control mice.

However, experiments utilising the interferon-resistant subline L1210R (108), suggest that a direct effect may not be interferon's only antitumour mechanism. Gresser et al. (105) found that interferon could protect mice against L1210R as well as L1210S tumour cells, suggesting some inhibitory effect mediated by some indirect mechanism. L1210S cells were, however, rather more sensitive than L1210R cells, so it is possible that interferon had some direct inhibitory effect. The difference may not have been significant however since such small numbers of mice were used (nine for each subline).

Evidence that the immune system may be involved comes from smears taken from P388 and L1210 leukaemias (246) and Ehrlich Ascites (EA) tumours in mice (91).

Samples taken from interferon-treated mice showed tumours heavily infiltrated with well-differentiated macrophages and phagocytosis of tumour cells was frequently seen. Samples of tumours from control mice showed few macrophages, which actually degenerated as the tumours grew, and phagocytosis of tumour cells was not observed (91).

In vitro and in vivo studies have shown that interferon can directly stimulate cytotoxicity and phagocytosis by lymphocytes and macrophages and activity of natural killer cells (84, 116, 212, 258). This is discussed in more detail later.

Other experiments suggest that there may be no involvement of the immune system. Interferon still inhibited growth of L1210 and EA tumours in mice that had been treated with antilymphocyte serum (ALS), X-irradiation (to inactivate lymphocytes) or silica (to inactivate macrophages), though the latter treatment reduced IFN's antitumour activity a little (93). Growth of L1210R, as well as L1210S, cells in treated mice was inhibited by interferon, so this suggests that either the ALS, X-ray and silica treatments did not fully suppress lymphocyte and macrophage activity, or some other host-mediated mechanism was not affected. The most likely candidate for the latter is natural killer cell activity.

Yokota et al. (281) have provided strong evidence that interferon may directly inhibit in vivo growth of tumours. Tumour formation by HeLa cells or human bladder carcinoma cells in nude mice was inhibited by human interferon, but not mouse interferon. Conversely tumour formation by mouse sarcoma 180 cells in nude mice was inhibited by mouse interferon, but not human. Similar experiments have been conducted by Taylor-Papadimitriou and Balkwill which generally support the findings of Yokota et al. (293).

Thus much evidence implicates both direct inhibition of tumour cell growth and a stimulated immune system, but the relative importance of each in different tumours and animals is still not known.

#### Inhibition of Growth in vitro

As already mentioned, interferon was, for some time, considered to be a purely antiviral substance, and effects on cellular behaviour were attributed to contaminants in the interferon preparations (19, 160, 182). Effects on cell morphology (89), cell growth (197) and RNA synthesis (233) were reported in the early 1960s, but it was not until the late 1960s and early 1970s that such effects were studied seriously, at about the same time as effects on tumour growth were observed.

Gresser and his colleagues demonstrated beyond reasonable doubt that interferon could indeed inhibit growth of cells. They used mainly L1210, a murine leukaemia line. Growth rate and maximum density of these cells, in suspension (101, 103, 104, 107, 172), was inhibited by a number of interferon preparations from different sources induced by different agents. In subsequent experiments with L1210 cells (100) they were the first to suggest that the growth inhibition may be cell cycle dependent. Cells seeded at high density were less sensitive to interferon than those seeded sparsely, and they found that the degree of growth inhibition was

directly proportional to the number of population doublings a control culture needed to go through to reach saturation density at the time of interferon treatment of test cultures.

Further proof for cell cycle dependence came from Macieira-Coelho et al. (172) who found that interferon decreased the rate of entry of newly subcultured L1210 cells into S phase, although all cells eventually entered it. Thus they concluded that the doubling potential of interferon-treated cells was reduced.

A subline of L1210 selected for resistance to interferon's antiviral action was found to be resistant to cell growth inhibition as well, thus strengthening the argument that interferon can inhibit cell growth. Many also found that the maximum cell density reached was reduced (5, 54, 81, 100, 119, 120, 121, 151, 152, 157, 166, 170, 172, 175, 190, 198, 244). Some studies have found that transformed cells were more sensitive than normal ones (143, 157, 166). For instance Knight (143) found that saturation densities of L cells and SV40- and polyoma virus-transformed 3T3 cells were greatly reduced, while those of nontransformed 3T3 and mouse embryonic fibroblasts were unaffected. On the other hand, others have found little correlation between sensitivity to growth inhibition and transformation (190).

Interferon also inhibited the ability of dispersed macrophages to grow into colonies in agar (180), and the phytohaemagglutinin (PHA)- or allogeneic cell - stimulated growth of lymphocytes (167).



With few exceptions, studies that have measured cell viability have found that interferon is not toxic to cells (54, 100, 143, 166). However, Tovey and Brouty-Boye (254), using Ll210 cells growing in a chemostat, found that the growth rate could be closely controlled by the rate of medium flow. Below a certain growth rate mortality increased and the culture died out. Interferon treatment (6400 U/ml) slowed growth of cultures by 1.8 fold, regardless of initial doubling time. The faster growing cultures showed no increase in mortality, but those growing more slowly started to die following interferon treatment. They proposed that interferon was not toxic per se, but cells will die if it reduces their growth rate below a sustainable level.

Whilst it remained impossible to completely purify interferon it was in doubt whether the growth inhibition was actually due to the interferon itself or to some contaminant. Gresser et al. found that in several mouse IFN preparations the ratio of antiviral to anticellular activities was constant, and in dose response curves, thermal inactivation curves and appearance and rise of activity in supernatants above NDV-induced L cells, antiviral and anticellular activities paralleled each other, showing that the two were inseparable (107). However, Lindahl-Magnusson et al. (166) found that mouse interferons from different sources could vary in their growth inhibitory potency, requiring differing numbers of antiviral units to cause a 50% inhibition of growth

of a given cell type.

Other experiments found that anticellular and antiviral activities of mouse and human IFNs were present in a constant ratio in different preparations when tried on neonatal foreskin fibroblasts (146) and Daudi lymphoblastoid cells (119, 120). The two activities were also inseparable by polyacrylamide gel electrophoresis (190), or by rigorous denaturation and renaturation by boiling in SDS (238). However, Borecky et al. (34) were able to separate the antiviral and anticellular activities of human interferon by electrophoresis. Dahl and Degre (55, 56) claimed to have separated human interferon into a protein component with antiviral activity, which bound to albumin, and a glycolipid with molecular weight of 3200 which contained the growth inhibitory activity. It was proposed that this glycolipid is actually held within the folded structure of the interferon protein molecule which has made it difficult to separate the two activities.

Purification of interferons to homogeneity has now ruled out the possibility that a contaminant is responsible for cell growth inhibition, since all purified interferons so far tested possess both antiviral and anticellular activities (58, 82, 174), though the possibility of a tightly-bound component as proposed by Dahl and Degre cannot be excluded.

#### Growth Inhibition Studied in Synchronised Cultures

Following suggestions that the growth inhibition may be

cell cycle dependent, a number of studies were conducted to identify whether any particular part of the cell cycle was more sensitive than the rest. Data so far obtained using  $^3\text{H}$ -thymidine incorporation into cells synchronised in Go by density dependent growth control or serum starvation (18, 54, 170, 231), at the G1/S boundary by thymidine block (79, 194), or in mitosis by colcemid (79), provide evidence suggesting that interferon treatment arrests cells in late G1, possibly at the Go-G1 boundary, but probably not as late as the G1-S boundary (170, 194, 231, 264). Some reports also suggest that there may be a block in G2+S phases in some cells (18, 54, 79, 175). It is quite possible that interferon may arrest different cell types at different stages of the cell cycle.

#### Other Effects on the Cellular Phenotype

a) Morphology Gresser's observation of a change in morphology of human amnion cells from epithelial to fibroblastic when exposed to interferon for three days, was one of the first non-antiviral effects observed (89). Chany and Vignal (48) isolated from a MSV-transformed BALB/c 3T3 line a revertant subline with normal morphology by cultivating in the presence of interferon for 200 passages. However, since the transformed line was a virus producer it is likely that interferon acted by inhibition of virus growth. More recently Fuse and Kuwata (78) found that transformed human RSa cells

changed from a fibroblastic to an epithelial morphology. This they equated with the observation that cells in S phase tended to be epithelial, while those in G1 were fibroblastic. Using cultures synchronised by thymidine they found that onset of the first S phase following release was not delayed by interferon, but mitosis started five hours later than in controls (27, 93). They concluded that the increase in epithelial-like appearance was due to the cells being delayed in S phase. However, the second S phase following release was almost completely abolished, suggesting that there was a major interferon-induced block in G1 following mitosis.

Since then there have been few reports of a change in cell morphology, though recently Pfeffer et al. (201-2) found that normal human fibroblasts became flatter, larger and organisation of the cytoskeleton and fibronectin matrix increased. In addition Bal de Kier et al. (14) found that interferon had no effect on mature mouse neuroblastoma cells, but stimulated immature cells to grow long processes from the cell body and take on the appearance of fully differentiated neuroblastoma cells. Linked to the morphology studies is the finding by Brouty-Boye and Zetter (39) that both mouse and human interferons could inhibit motility of homologous normal and transformed cells.

b) Alterations to biosynthesis High doses of interferon can cause a general inhibition of RNA and protein synthesis,

up to 50% (ref 236 p242), but more significantly there are effects on synthesis of specific products, which may be stimulated, inhibited or even both. Low doses of interferon can stimulate its own production following viral induction (priming), and can also stimulate haemoglobin synthesis in DMSO-stimulated Friend erythroleukaemia cells (63, 214). High doses inhibit synthesis of both (213, 214, ref 236 p236). Similarly very low doses of interferon can stimulate, while higher doses inhibit, antibody production.

Histamine synthesis by leukocytes exposed to ragweed antigen E or anti IgE is stimulated by interferon (ref 236 p249), and it can also stimulate synthesis of prostaglandin E by human synovial and foreskin fibroblasts (ref 236 p248).

Production of several enzymes is inhibited; for instance dexamethasone-induced tyrosine amino transferase in rat hepatoma cells (ref 236 p243).

The physiological significance, if any, of all these changes is unknown, though fever, a side-effect of interferon treatment, may be due to production of histamine and prostaglandin E.

c) Cell surface changes Interferon has a whole host of effects on cell surfaces ranging from effects on overall morphology to the expression of certain specific antigens.

The transport of various nutrients may be changed. Tovey and his colleagues (38, 255) found that uptake of <sup>3</sup>H-thymidine

into the acid soluble pool of L1210 cells grown in a chemostat could be inhibited within two hours of initiation of interferon treatment, whereas cell growth rate remained unchanged until 18-22 hours later. O'Shaughnessey et al. (194) also recorded that uptake of  $^{14}\text{C}$ -amino acids and  $^3\text{H}$ -thymidine into L cells was inhibited by interferon. Secretion of plasminogen activator (PA) from SV40-transformed 3T3 cells was inhibited by IFN by 58% without having any effect on intracellular PA levels (223). High IFN doses did inhibit PA synthesis, but only in line with a general reduction in RNA and protein synthesis. This report is especially significant since it is one of the few which clearly demonstrates an inhibitory effect on a transformation-specific phenomenon, and a phenomenon which, as outlined earlier, may be important to the transformed phenotype.

Binding of  $^{63}\text{Ni}$ -Con A to the surfaces of L1210 cells is stimulated by interferon treatment (124), but there are no reports of an effect on the agglutinability of transformed cells. Increased binding may be due to increased expression of surface antigens. The expression of histocompatibility antigens on L1210 cells in vitro is stimulated, as is expression on mouse splenic lymphocytes and thymocytes in vivo (164, 165, 168, ref 236 p249). Expression of carcinoembryonic antigen (CEA) on surfaces of human colon carcinoma cells is also enhanced by interferon (10). Such enhancement of expression may aid the host defences to recognise foreign cells. In

addition to these increases in antigen expression, Chang et al. (47) using freeze fracture techniques, observed that interferon induced an increase in the density of intramembranous particles in mouse AKR cells.

#### Immune Effects of Interferon

a) Antibody production Interferon generally inhibits antibody production, but under some circumstances its synthesis may be stimulated. For instance, 80 units/ml inhibited antibody production by mouse lymphocytes when exposed to sheep red blood cells (SRBC) - a T cell-dependent antigen - but 0.8 units/ml stimulated antibody synthesis. In vivo injections have had a similar effect (ref 236 pp254-256).

Production of antibody in mice in response to the B cell-dependent antigen, Salmonella typhimurium lipopolysaccharide, could also be inhibited by interferon injection (ref 236 p255).

b) Lymphocyte cytotoxicity Incubation of sensitised lymphocytes with interferon for a few hours can greatly enhance their cytotoxicity towards specific target cells (116, ref 236 p250). This specific cytotoxicity is mediated by T lymphocytes. Non-specific or spontaneous cytotoxicity towards foreign cells, to which the immune system has not previously been exposed, is mediated by Natural Killer (NK) cells. These are non-B non-T lymphocytes which are equally

active in athymic nude mice as in normal mice (84, 112, 212). Both in vitro and in vivo interferon has had a direct stimulatory effect on NK cell cytotoxicity (64, 84, 116), possibly by recruitment of previously inactive pre-NK cells (217).

Interferon treatment of the target cells instead of the lymphocytes does not stimulate specific nor spontaneous cytotoxicity, but may actually render the cells resistant to the lymphocytes (64, 67, 183, 184). Trinchieri and Santoli (258) found that while treatment of normal cells with interferon rendered them resistant to lymphocytes, interferon-treated transformed cells remained sensitive.

c) Macrophage activity Interferon, in vivo or in vitro, can increase cytotoxicity of macrophages to any allogeneic cells and syngeneic tumour cells. It can also increase macrophage phagocytosis of carbon particles and latex beads (ref 236 p251). The clearance of injected leukaemia cells and antibody-coated erythrocytes from the serum of mice is also greatly enhanced by interferon treatment (114, ref 236 p251).

Furthermore, in vitro macrophages will spread out more on a glass surface if treated with interferon than will untreated controls (221, ref 236 p252).

Neither the mechanisms nor the physiological significance of most of IFN's effects on fibroblasts, epithelial cells and



immunological cells is at present understood. Clearly, from these experiments, interferon has considerable effects on cell-mediated immunity which could be important in cytotoxicity and phagocytosis of foreign cells. Effects on both normal and transformed cells in vitro involve increasing antigenicity of both, which could presumably aid immune recognition, and slowing cell growth and division which in vivo would be most effective against rapidly growing tumour cells. Some further evidence, such as effects on morphology, saturation density and secretion of plasminogen activator, suggests that interferon may also partially revert the transformed phenotype.

Current knowledge of the mechanisms of growth inhibition and effects on the transformed phenotype are discussed below.

#### Possible Mechanisms for Interferon's Cellular Activities

The lack of understanding concerning the mechanisms of interferon's cellular activities contrasts sharply with that of the antiviral effect which is now quite well understood. There is still some question as to whether the effects on cellular metabolism could be mediated by the same or a similar mechanism to the antiviral effect, though most evidence would suggest not. However, a brief outline of the antiviral mechanism will be given here as a background to some of the anticellular studies.

a) The antiviral mechanism The kinetics of the development of the antiviral state following interferon treatment vary greatly between different cell lines, but generally speaking a one hour interferon treatment usually leads to the antiviral state being detectable three to four hours after treatment, peaking at eight to twelve hours, lasting for fourteen to twenty-four hours and declining slowly, tailing off for up to seventy-two hours until no viral resistance remains (ref 236 pp196-200).

There is evidence that interferon can inhibit virus replication at several stages of the infectious cycle, most viruses apparently being sensitive at at least one stage. The initial establishment of infection, particularly by retroviruses, can be blocked (71, 185, 200), as can assembly, maturation and release of these and other viruses from infected cells (25-27, 63, 76, 77). Some viruses, most notably SV40 and vesicular stomatitis virus (VSV) are sensitive to transcription inhibition (ref 236 p209).

The main block in replication appears to be in the translation of mRNA, and it is this which has been most extensively studied. In vitro a protein was identified in extracts of interferon-treated cells, but not controls, which could be activated by double-stranded RNA and ATP to synthesise a small molecule which itself activated an endonuclease and a protein kinase (11, 12, 13, 15, 16, 69, 142a, 208, 222, 270, ref 236 p214). This small molecule was identified as a novel

oligonucleotide pppA2'p5'Ap5'A (abbreviated to 2.5A). Thus the protein which synthesises 2.5A is termed 2.5A synthetase. It remains inactive until virus infection in vivo or dsRNA treatment in vitro (11, 16, 17, 123, 142).

The protein kinase, activated by 2.5A, phosphorylates at least two proteins - the alpha subunit of eIF 2 (a protein synthesis initiation factor) and a ribosomal protein (218, 219). This may inhibit the formation of the met.tRNA-mRNA-40s ribosomal subunit complexes prior to start of translation (162, ref 236 p216). This presumably frees the mRNA into the cytoplasm where it can be digested by the 2.5A-activated endonuclease. Both cellular and viral mRNAs are digested by this endonuclease, though reovirus mRNA is digested more efficiently than cellular mRNA (69, ref 236 p214).

The limited host range of interferon activity is determined at the cell surface, not by the 2.5A-activated system which has been found to be identical in a number of species (16). This is strengthened by observations by Blalock and his colleagues (28-31, 125), that once the antiviral state has been established in some cells it can be transferred between cells in the absence of interferon and even in the presence of interferon antiserum. This transfer can not only occur between homologous cells but also between cells of heterologous species. These observations argue for the transfer of a common antiviral agent between cells via intercellular connections.

b) The cellular phenotype Serum arrested or density inhibited cells are thought to rest in Go phase (or the A-state). The observations that interferon apparently arrests cell growth in this phase has led to speculation that its role may be to make cells more sensitive to a normal growth control mechanism (121, 172, 201). That increasing the serum concentration inhibits interferon's action (100), as well as stimulating serum starved or density inhibited cells to grow, strengthens this possibility. The mechanisms whereby these controls act are not known, let alone how interferon might affect their expression. Most studies of interferon's growth inhibitory mechanism have to date concentrated on trying to determine whether or not the antiviral mechanism may be involved in any way.

Numerous reports have implicated cyclic nucleotides as being activated by cell surface bound interferon to trigger both the antiviral and anticellular activities (80, 256, 265, ref 236 p194). The most popular candidate is cyclic AMP, though its involvement has been refuted. Tovey *et al.* (256), using L1210 cells grown in a chemostat found that interferon treatment was followed by a rapid rise in intracellular cyclic GMP, but cAMP levels did not rise until several hours later, more as a consequence than a cause. Fuse and Kuwata, however, did observe a correlation between increased cAMP levels following interferon treatment of human RSa cells and inhibition of cell growth (80).

Even if both activities do use a common second messenger it does not necessarily imply that they share a common mechanism; the second messenger may activate a cascade phenomenon affecting a whole range of processes. There have been a number of studies to identify whether the ratio of the anticellular and antiviral activities of interferon preparations on a range of cell types, is constant or varies. If it remains constant this would suggest that the mechanisms may be similar, but a variable ratio suggests independent mechanisms. Virtually all studies have found that the anti-cellular activity, usually measured as growth inhibition, is less potent than the antiviral activity, and that the ratio remains constant for different preparations on the same cell type, suggesting that the growth inhibition is at least a specific interferon activity (as discussed earlier) (107, 119, 120, 146, 238). However, when it comes to studying any one preparation on different cell lines there is disagreement. Some find that the ratio always remains constant (119, 237), while others find that the ratio varies (151, 152, 157, 166, 176, 186), so no clear-cut answer to this question has been possible.

Several interferon-resistant sublines have been selected. Both the L1210R subline, selected for resistance to interferon's antiviral activity (100, 101, 105, 108), and the transformation revertant of MSV-BALB (48), selected for resistance to the growth inhibitory activity, were resistant

to both antiviral and anticellular effects. The major difference between the two was that LI210R cells did not bind interferon on their surfaces, and so presumably were resistant for that reason, whereas the MSV-BALB revertant bound normal amounts of interferon and thus must have been resistant due to some intracellular mechanism. A third sub-line isolated by Kuwata et al. (151) was resistant to the growth inhibitory action, but was still as sensitive as the parental line towards antiviral activity. line

Hovanessian and Wood (122) recently treated cells with 2.5A at concentrations of 10-100 nanomolar. At low concentrations transient inhibition of DNA, RNA and protein synthesis was observed, but at high concentrations the effects were more persistent and were accompanied by cell growth inhibition. This high concentration also inhibited viral replication by 90-100%, but the physiological relevance of these amounts of extracellular 2.5A to intracellular levels normally synthesised as a response to interferon treatment and virus infection is not known.

An argument against the anticellular activity using the same mechanism as the antiviral activity is that of kinetics. The antiviral state takes only a few hours to develop after a very short exposure to interferon, whereas anticellular activities, including cell growth inhibition may take 20 hours or more to appear with continuous interferon treatment. However, it cannot be ruled out that the anticellular effect

may be the result of the cell being in a prolonged antiviral state.

Genetic studies, using human x mouse hybrids identified some time ago that human chromosome 21 determines sensitivity to both antiviral and anticellular activities of alpha, beta and gamma interferons (248, ref 236 p185). The equivalent mouse chromosome has only recently been identified as chromosome 16 (163). The gene(s) appears to code for the cell surface receptor (209), and <sup>the behaviour of the L1210R subline</sup> is presumably due to a defect in the gene(s) on chromosome 16, whereas that of the MSV-BALB revertant is due to a change in the genes coding for the intracellular mechanisms.

#### Reversion of the Transformed Phenotype

Most evidence presently available, which has been discussed in Part 2, indicates that interferon's antitumour activity may act principally by two routes, viz direct cell growth inhibition and stimulation of the immune system. Scattered evidence also suggests that interferon may cause transformed cells to behave in a more normal manner, but little attention has so far been paid to this possibility. Most of the experiments that have brought forward such evidence have already been mentioned in the context of interferon's general growth inhibition and effects on cell behaviour, but they are all brought together here simply to illustrate how sparse the presently available data are.

Knight (143) found that saturation densities of L cells and polyoma- and SV40-transformed 3T3 cells were reduced by interferon treatment to levels similar to the normal 3T3's. Saturation density of the 3T3's was unaffected. These results suggest that the transformed cells regained some density dependent growth inhibition. Several other reports have found that transformed lines may be more sensitive to growth inhibition by interferon than are normal cells (86, 157, 244). Bourgeade and Chany (35) found that sodium butyrate enhanced the antiviral and cell growth inhibitory activities of interferon on MSV-transformed mouse BALB/c and Syrian hamster cells, but not on normal cells. They suggested that this may be due to the elaboration of the microfilament and microtubule systems induced by butyrate in transformed cells.

Gresser et al. (103) was able to inhibit tumour formation in vivo or colony formation in agar by L1210 cells by pre-treating them in vitro for several days with interferon. This may be due to a reversion of their phenotype, but it could also be due to residual growth inhibition.

The most clear-cut effect on a transformation-specific phenotype is that observed by Schroder et al. (223) where interferon inhibited secretion of plasminogen activator from SV40-transformed 3T3 cells.



All these observations, though they suggest that interferon may actually revert the transformed phenotype, are far from unequivocal proof. No systematic study has yet been undertaken to assess whether reverse transformation by interferon is possible, despite the fact that such an effect could have profound implications for our understanding of the antitumour activity. The aim of this project, therefore, is to carry out such a systematic study and establish whether or not interferon really can cause reverse transformation.

## MATERIALS AND METHODS

### MATERIALS

General chemicals that are commercially available were usually of 'Analar' grade, and were supplied by BDH, Fourways, Atherstone, Warwickshire, and by Fisons of Loughborough, Leicestershire. Named reagents were supplied as follows:

#### BDH Chemicals, Fourways, Atherstone, Warwickshire

Ethylenediaminetetraacetic acid (EDTA)

Fluorescein isothiocyanate (FITC)

Beta-mercaptoethanol

#### Sigma (London) Chemical Co. Ltd., Poole, Dorset

Bovine serum albumin (BSA)

Concanavalin A

Cyclohexylaminopropanesulphonic acid buffer (CAPS)

Deoxyribonuclease I

Gelatin

#### Fisons, Loughborough, Leicestershire

1,4-Dioxan (spectroscopic grade)

Folin and Ciocalteu's phenol reagent

#### Aldrich Chemical Company Ltd., Gillingham, Dorset

Butyric acid

Collaborative Research, Waltham, Massachusetts, U.S.A.

Human fibronectin

Rabbit anti-human fibronectin antiserum

Wellcome Reagents Ltd., Hither Green Lane, London SE13

FITC conjugated goat anti-rabbit IgG

Gibco Bio-Cult Ltd., Washington Road, Paisley, Scotland

Freund's complete and incomplete adjuvants

Pharmacia Fine Chemicals, Uppsala, Sweden

Poly U-Sepharose 4B

Blue-Sepharose CL6B

Sephadex G25 and G50

Cyanogen Bromide-activated Sepharose

Bio-Rad Laboratories Ltd., Holywell Industrial Estate,

Watford, Herts.

Affi-gel 202

George T. Gurr, Searle Scientific Services, High Wycombe, Bucks.

Crystal Violet

Flow Laboratories Ltd., Irvine, Ayrshire

Newborn calf serum

Dulbecco's and Glasgow's modifications of Eagle's medium

(DMEM and GMEM)

Versene

GMEM, ten times concentrated

Glutamine

Difco Laboratories, East Molesey, Surrey

Noble Agar

Glaxo Laboratories Ltd., Greenford, Essex

Penicillin and streptomycin

Radiochemical Centre, Amersham, Bucks.

<sup>3</sup>H-uridine (26 Ci/mMol)

<sup>3</sup>H-thymidine (25 Ci/mMol)

<sup>3</sup>H-leucine (342 Ci/mMol)

<sup>35</sup>S-methionine (740 Ci/mMol)

Packard Instrument Co. Inc. U.S.A.

Insta-gel scintillation cocktail

Eastman-Kodak, Rochester, New York, U.S.A.

N,N,N',N'-Tetramethylethylenediamine (TEMED)

Panatomic-X 35mm film roll

Tri-X Pan 35mm film roll

Photo Flow wetting agent

X-omat-H X-ray film

Kodafix fixer

Ilford Ltd., Basildon, Essex

Ilfospeed multigrade filters

Ilfospeed multigrade printing paper

Ilfospeed multigrade developer

Hypam rapid fixer

Paterson Products Ltd., Boswell Court, London

Aculux and Acutol film developers

#### METHODS

##### Cells

Normal mouse fibroblasts used were continuous lines of C3H and NIH mouse embryo fibroblasts, C3H10T $\frac{1}{2}$  cl8 (210) and NIH 3T3cl4E (289, 290), and primary cultures of NIH and C3H mouse embryo fibroblasts (MEF) prepared in this laboratory. The established lines, although aneuploid and capable of indefinite growth in vitro, were nevertheless normal by the criteria of anchorage dependence and failure to form tumours in syngeneic hosts.

Kirsten Sarcoma Virus-transformed clones were derived from C3H10T $\frac{1}{2}$ , NIH 3T3 4E and NIH MEF; MSV C3H2 and 6 (from C3H10T $\frac{1}{2}$ ); CC1 and cA12 (from NIH 3T3 4E) and MSV MEF2 and 4 (from NIH MEF). These transformed cells were non-producers with the exception of MSV C3H6 which produced both KiSV and KiLV.

All cell types were cultured in Dulbecco's modification of Eagle's medium supplemented with 10% new-born calf serum.

Confluent cells were passaged by trypsinisation with 0.2% trypsin plus 0.02% EDTA and recultured at 1:8 dilution so that each passage was equivalent to roughly three population doublings.

After approximately 20 passages the C3H10T $\frac{1}{2}$  cl8 and NIH 3T3 4E cells were discarded and new vials of early passage cells thawed from liquid nitrogen, since after that many passages spontaneous transformation started to occur.

Despite disadvantages of mouse cells caused by their instability and tendency to spontaneously transform, there were several reasons for choosing to use cells of this species -

- a) clones of mouse fibroblasts transformed by Kirsten Sarcoma virus were already being studied in this laboratory to establish numbers and locations of proviral integration sites. Thus several clones were immediately available and transformation by KiSV to produce further clones was possible;
- b) most of the early studies of interferon's anticellular and antitumour activities had been conducted in mice and it was therefore appropriate to extend these observations;
- c) mouse interferon was available in much larger quantities than human interferon, since L cells grew more readily and were much more prolific producers of interferon than any human cells that were available;
- d) it would be possible to transplant mouse cells into syngeneic hosts for in vivo tumourigenicity studies if necessary.

#### Interferon Production and Purification

Initially a series of studies was conducted to improve yields of crude interferon and the final purity of that

interferon following purification steps. Those studies are described in chapter one of the Results. Here is described the final method adopted for production of interferon.

Mouse interferon was produced routinely from confluent monolayers of L cells by induction with Newcastle Disease Virus (NDV). The cells were grown in roller bottles in Glasgow's Modification of Eagle's medium supplemented with 10% new-born calf serum. When confluence was reached fresh medium was added. Twenty-four hours later the medium was removed and 5 mls of NDV (HAU:  $10^4$  units/ml) added to each bottle for one hour. After this period excess virus suspension was removed and 50 mls of serum-free GMEM added.

The cells were incubated for 16 hours and then the interferon-containing medium was harvested and acidified by HCl to pH 2. The crude interferon was left at pH 2 at  $4^{\circ}\text{C}$  for 7 days. Each batch yielded roughly 500 mls of crude interferon containing  $5 \times 10^7$  to  $5 \times 10^8$  units. After seven days the solution was dialysed against 10mM Tris-HCl, pH 7.5 for 48 hours. The dialysed interferon was then applied to a column of Blue-Sepharose CL-6B which had been equilibrated with the same buffer. After passing the crude solution through, the column was washed with 10mM Tris-HCl, pH 7.5, and the interferon then eluted with the same buffer containing 1M NaCl and 8M urea. The IFN-containing eluate was then dialysed against 10 mM Tris-HCl, pH 7.5 for 24 hours and

applied to poly U-Sepharose 4B, equilibrated with the same buffer. After application of the interferon and washing of the column, the interferon was eluted with 10mM Tris-HCl pH 7.5 containing 1M NaCl.

Final yields of IFN were usually approximately 50-80% of starting material in about 5 mls of eluate. The specific activity of batches obtained by this technique varied between  $5 \times 10^7$  units/mg of protein and  $2 \times 10^8$  units/mg of protein.

#### Assay of Interferon Titres

Interferon was assayed in L cells by the viral RNA synthesis reduction assay (286), using Semliki Forest Virus (SFV) as challenge, or by plaque reduction, using Encephalomyocarditis (EMC) virus as the challenge virus. Titres for the same interferon sample were roughly equal when assayed by the two methods.

One unit in these assays was equal to one international reference unit.

#### Measurement of Protein Content

The standard procedure used was the method described by Lowry et al. (169), though during experimental studies to improve interferon purification (chapter one of Results), an assay using fluorescamine, described by Bohlen et al. (33), was often used.



Bohlen et al claimed that the fluorescamine assay was accurate at protein concentrations in the nanogram range. Fluorescamine reacts with primary amines to form fluorescent complexes. The reaction proceeds at room temperature in aqueous solution at pH 8-9 with a reaction half-time in milliseconds. Unreacted fluorescamine is broken down to water-soluble products. Fluorescamine itself is insoluble in aqueous solution so it has to be dissolved in an organic solvent which gives minimal absorbance, such as acetone or spectroscopic grade 1,4-dioxan.

The manual procedure described by Bohlen et al. was followed, except that 1.0 ml of the fluorescamine solution was used instead of 0.5 ml. It was found that the former gave more reproducible results. The procedure was as follows. Fluorescamine was dissolved in 1,4-dioxan at a concentration of 30 mg/100 mls. 100  $\mu$ ls of the protein sample was made up to 1.5 mls with 0.05 M sodium phosphate buffer at pH 8. 1 ml of fluorescamine was then added, while the sample was shaken on a vortex mixer. The mixture was then left for 5 minutes for fluorescence to reach its maximum, which was then measured in a Perkin-Elmer MPF3 spectrofluorimeter with excitation at 390 nm and emission at 475 nm. Using bovine serum albumin as standard a linear relationship between fluorescence and protein concentration was obtained in the range 20-100  $\mu$ g/ml. Below 20  $\mu$ g/ml reproducible results could not be obtained.

Measurement of Antiviral and Anticellular Activities in  
Different Clones

Sensitivities of C3H10T $\frac{1}{2}$  cl8, NIH 3T3 4E and several KiSV-transformed clones towards interferon's antiviral activity were measured using the viral RNA synthesis reduction assay with SFV as challenge virus. Transformed cells were seeded at  $2 \times 10^5$  cells per vial and normal cells at  $5 \times 10^4$  cells per vial. Interferon doses used were in the range of  $10^4$  to  $10^{-3}$  units/ml. Sensitivity was determined from the dose response curves as the interferon dose required to inhibit viral replication by 50% (VI50).

To measure the sensitivities of these cells to cell growth inhibition 2000 cells were seeded per well of a 96 well microtitre tray and incubated for 24 hours in DMEM supplemented with newborn calf serum. Fresh medium was then added containing interferon at concentrations ranging from  $10^4$  to  $10^{-3}$  units/ml. After a further 72 hours, the cells were incubated for 3 hours in the presence of  $^3\text{H}$ -thymidine (10  $\mu\text{Ci/ml}$ ). Radioactivity in the acid insoluble material was then determined by solubilising cells in 1 M NaOH, neutralising in 1 M HCl and mixing an aliquot in instagel scintillation cocktail, and then counting in a Packard liquid scintillation counter at 12% gain. Sensitivity was determined from the dose response curves as the dose of interferon that would inhibit cell growth by 50% (GI50).

DNA Synthesis at Different Cell Densities

Cells were seeded into wells of microtitre trays at a

range of densities, 500-20000 cells per well in DMEM supplemented with 10% newborn calf serum. After 24 hours incubation interferon at  $10^4$  units/ml was added to half the wells. In some experiments butyric acid was added to half the wells to measure its effects on DNA synthesis alone and in the presence of interferon.

After a further 24 hours  $^3\text{H}$ -thymidine ( $10 \mu\text{Ci/ml}$ ) was added for three hours, and radioactive incorporation into the acid insoluble material determined.

#### Growth Curves and Saturation Density

$3 \times 10^4$  cells were seeded per 35 mm Petri dish in DMEM supplemented with 10% newborn calf serum. After 24 hours interferon ( $10^4$  units/ml) was added to half the cultures. Fresh medium and interferon were added every day to ensure growth was not nutrient-limited. At intervals pairs of dishes were trypsinised and cells counted with a haemocytometer. The means of cell numbers in each pair of dishes were recorded.

In some experiments butyric acid (0.5 mM) was also present to determine its effects on growth curves and saturation density when alone and in the presence of interferon.

#### Focus Assay

$10^2$  transformed and  $10^5$  normal cells of the parental cell type were seeded together in 50 mm Petri dishes in DMEM supplemented with 10% newborn calf serum. Twenty-four hours

later this medium was replaced with DMEM supplemented with 5% heat-inactivated newborn calf serum. IFN ( $10^4$  U/ml) was added to half the dishes at this point. Thereafter, fresh medium and IFN were added every third day until foci formed by the transformed cells were clearly visible on the control plates at which time foci on all plates were scored, usually at about 10 days.

#### Anchorage Dependence

$10^3$  transformed cells suspended in DMEM supplemented with 20% newborn calf serum and 0.25% agar were overlaid onto a base of this same medium containing 0.4% agar (173) in 50 mm Petri dishes. For IFN treated cultures, IFN was added to both the overlay and the base layer at  $10^4$  U/ml. Colonies were counted, after 28 days incubation, on a low power microscope.

#### Cloning Efficiency in Liquid Medium

$10^2$  cells were seeded per 50 mm Petri dish in DMEM supplemented with 20% newborn calf serum. After 24 hours IFN at  $10^4$  units/ml was added to half the dishes, and thereafter fresh medium and IFN were added every third day until colonies were clearly visible. The plates were then fixed in methanol and stained with crystal violet, and the colonies counted on a low power microscope.

### Concanavalin A Agglutination

Cells were grown in 50 mm Petri dishes in DMEM supplemented with 10% newborn calf serum  $\pm$  interferon until saturation density was reached. The cultures were then rinsed and cells removed by 0.02% EDTA in phosphate buffered saline (PBS). Trypsin was not used since trypsinisation of cells increases their agglutinability. The cells were centrifuged and then resuspended in PBS at  $10^6$ /ml. Two millilitre aliquots of this suspension were added to siliconised glass flasks. Concanavalin A (100  $\mu$ g/ml) was added to half the flasks, which were then put on a shaking water bath at 37°C. Samples were taken at intervals and cells not agglutinated were counted with a haemocytometer.

### Morphological Studies

Cells were grown in 35 mm Petri dishes with DMEM supplemented with 10% newborn calf serum for up to a week with or without interferon up to  $10^4$  units/ml. Normal cells were usually fixed at or just before confluence, and transformed cells were fixed while they still formed a single layer over most of the dish. In some experiments butyric acid (0.5 mM) was also added to observe its effects on morphology when alone and when in the presence of interferon. Three parameters were studied;

- i) Cell and culture morphology Cells grown in 35 mm Petri dishes were fixed for 20 minutes at room temperature with

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- i) Cell and culture morphology Cells grown in 35 mm Petri dishes were fixed for 20 minutes at room temperature with

3.5% formaldehyde in PBS and then stained with crystal violet. The cultures were examined at low power on a Leitz Diavert inverted microscope.

ii) Microfilament system Cells grown on glass coverslips were fixed for 20 minutes at room temperature with 3.5% formaldehyde in PBS and then treated with methanol at  $-20^{\circ}\text{C}$  for 15 minutes to make the plasma membranes permeable. The coverslips were washed repeatedly with PBS and then stained with deoxyribonuclease I to which was coupled fluorescein isothiocyanate (FITC) for 45 minutes at  $37^{\circ}\text{C}$ . DNase I binds specifically to actin (32), as well as DNA, and thus FITC-DNase gives a quick and simple method to label the actin microfilament system (118, 261). The stained coverslips were washed in several changes of PBS for  $1\frac{1}{2}$  hours and then mounted on slides in PBS-glycerol (1:9). The cells were examined under a Reichert Zetopan UV fluorescence microscope.

iii) Fibronectin pattern Cells grown on coverslips were fixed with formaldehyde as above, but since fibronectin is a surface protein the cells were not permeabilised with methanol. In some experiments live cells were treated with 1% NP40 in PBS for one hour immediately prior to fixation. This treatment removed the cells but the matrix between cell and substrate remained.

Rabbit anti-fibronectin antiserum was applied to all coverslips for 30 minutes at  $37^{\circ}\text{C}$ . The antiserum used was either a commercial preparation (Collaborative Research)

directed against human cell-surface fibronectin, or a preparation made in this laboratory directed against fibronectin purified from newborn calf serum.

After labelling with anti-fibronectin the coverslips were washed with PBS and then stained for 30 minutes at 37°C with sheep anti-rabbit IgG conjugated with FITC. Stained coverslips were then washed in PBS for 1½ hours and then mounted on slides in PBS:glycerol (1:9). The Reichert fluorescence microscope was again used.

#### Conjugation of FITC to DNase I

The conjugation procedure used was similar to that described by Johnson and Holborrow (136). Basically, dry FITC was added to a solution containing 10 mg of DNase I per millilitre of buffer, in the ratio of 20-40 µg FITC/mg of DNase. The buffer in which the DNase I was dissolved was 0.15 M NaCl and 10% by volume of carbonate buffer (5.8 ml of 5.3% Na<sub>2</sub>CO<sub>3</sub> and 10 mls 4.2% NaHCO<sub>3</sub>, adjusted to pH 9.5). The mixture was then shaken gently for 2 hours at room temperature during which time the FITC slowly dissolved in the solutions and ensured a slow binding of FITC to DNase, decreasing the of risk over-labelled DNase molecules. Conjugated FITC-DNase I was then separated from free FITC by gel filtration on Sephadex G50 medium grade, the conjugated material being washed through by PBS ahead of the free FITC.

Usually, 10-50 mg DNase I were conjugated at any one time,



and this could be collected in a final volume of PBS of 2-10 mls and then stored at  $-20^{\circ}\text{C}$ . These FITC-DNase I fractions could usually be diluted up to 1:2 with PBS before use to stain cells.

#### Coupling of Affinity Adsorbents to CNBr-Activated Sepharose 4B

Bovine Serum albumin (BSA) and gelatin were coupled to CNBr-activated Sepharose 4B for studies on interferon purification and the purification of serum fibronectin from newborn calf serum respectively. In each case the method used was the same. 2-5 gm of activated Sepharose was soaked and then washed for 15 minutes in 1mM HCl. This treatment swelled the powder into a gel and washed out dextran and lactose which are added to the Sepharose to preserve its activity when freeze-dried. One gram of powder gave approximately 3.5 mls of gel.

Immediately after swelling, the protein to be coupled - either BSA or gelatin - was added to the gel at a concentration of 5-10 mg/ml gel, dissolved in an alkaline coupling buffer (0.1 M  $\text{NaHCO}_3$  pH 8.3, containing 0.5 M NaCl). The alkaline pH maintains the amino groups in the unprotonated form and thus apparently aids binding of the protein to the Sepharose. The high salt content prevents protein-protein adsorption which thus also aids coupling to the Sepharose.

The coupling was allowed to proceed for 2 hours at room temperature, and then excess active groups on the Sepharose

gel were blocked by suspending the gel in 1 M glycine or tris buffer for 2 hours at pH 8.

The final gel was then washed alternately four or five times with acetate buffer (0.1 M, pH 4), and carbonate buffer (0.1 M, pH 8), each containing 0.5 M NaCl.

This washing procedure removes excess non-covalently bound protein and any other contaminating agents, but does not affect covalently bound material.

The BSA-Sepharose 4B and gelatin-Sepharose 4B were then stored at 4°C in PBS containing sodium azide until use.

#### Fibronectin Purification

Serum fibronectin was purified from newborn calf serum by the method described by Engvall and Ruoslahti (68). Basically, serum which had been dialysed for 48 hours against PBS, pH 7.2, containing 0.01 M sodium citrate was applied to a column of gelatin-Sepharose equilibrated in the same buffer. Adsorbed material was then eluted with 8 M urea in 0.05 M tris-HCl at pH 7.5. The fibronectin was removed immediately and could be collected in 2 x 2 ml fractions. Protein content in the fractions was estimated by absorption at 280 nm and the Lowry method. Polyacrylamide gel electrophoresis, using Collaborative Research human fibronectin as marker, showed this fibronectin to be contaminated by several smaller proteins, most of which could be removed by gel filtration on Sephadex G200. One protein which ran slightly slower than fibronectin remained just visible.

#### Preparation of Antiserum to Fibronectin

Purified serum fibronectin was used to prepare antisera from New Zealand White rabbits. For the first injection 0.5 mg fibronectin, dissolved in 0.5 mls PBS (pH 7.2) was mixed with an equal volume of Freund's complete adjuvant, and then injected subcutaneously. Further injections of 0.5 mg fibronectin with incomplete adjuvant were given every 2 weeks thereafter. The rabbit was first bled from the ear six weeks after the first injection and fortnightly thereafter. Appreciable amounts of antisera were produced eight weeks after the first injection.

The antiserum was tested, along with Collaborative Research anti-human fibronectin antiserum against purified human cell and calf serum fibronectin and the sera of calf, horse, mouse and chick, by Ochterlony diffusion immunoprecipitation.

#### Attachment of Cells to Glass

Cells were grown in Petri dishes until dense as described above, and were then fed medium containing  $^3\text{H}$ -leucine (5  $\mu\text{Ci}/\text{ml}$ ) for 24 hours, followed by a five hour period with no radioactive leucine. At the end of this time the cells were trypsinised and resuspended at  $10^5$  cells/ml in serum-free medium. 0.5 ml aliquots were then added to glass scintillation vials which had previously been soaked for 2 hours with 0.5 ml of PBS or fibronectin purified from newborn calf serum (100  $\mu\text{g}/\text{ml}$ ).

After one hour the cell suspensions were removed, the vials lightly rinsed once with PBS and then instagel scintillation cocktail added to measure radioactivity which was proportional to the number of adsorbed cells.

#### Polyacrylamide Gel Electrophoresis of Cell Lysates

Cells were seeded in 35 mm Petri dishes at  $3 \times 10^4$  cells per dish in DMEM supplemented with 10% newborn calf serum. Twenty-four hours later interferon ( $10^4$  U/ml) was added to half the dishes. After several days incubation, but while still subconfluent, the medium was removed and replaced with methionine-free medium with or without interferon, and incubated for a further six hours. At the end of this period half the dishes were trypsinised and cells counted with a haemocytometer, while in the other half the medium was removed, cells washed with PBS and 100  $\mu$ l PBS containing 100  $\mu$ Ci  $^{35}$ S-methionine added to each dish. After 45 minutes incubation the radioactive methionine was removed, dishes washed with PBS and cells solubilised by 2% SDS and 1% mercaptoethanol in 5 mM tris buffer (pH 9). 2  $\mu$ l aliquots were removed from each sample and washed on scintered glass filters with 5% trichloroacetic acid, followed by ethanol and diethyl ether, and then air dried. The dried filters were put in glass scintillation vials, solubilised with toluene/PPO scintillation cocktail and the  $^{35}$ S-methionine incorporated counted.

Of the remaining 98  $\mu$ l of each sample, 40  $\mu$ l was taken

and diluted to 50  $\mu$ l with the same buffer as was used to solubilise the cells, containing bromophenol blue.  $5 \times 10^5$  counts per minute of each sample were applied to polyacrylamide gels, as described by Laemmli (153), with a 4% stacking gel and 8% resolving gel.

X-Omat H film was used to visualise the protein bands. Fibronectin and actin were then quantitated by cutting out the respective bands in the gels, extracting the protein into 3 mls of a scintillant, at 37°C for 48 hours, and counting  $^{35}\text{S}$  in a liquid scintillation counter. The scintillant consisted of (in a 2 litre volume) : NCS tissue soluboliser (180 mls), liquifier ( 8 g PPO + 160 mg POPOP in 84 mls of toluene), toluene (1716 mls) and water (20 mls).

#### Immunoprecipitation of p21

In this experiment cell cultures were handled by myself, but labelling of cultures with  $^{35}\text{S}$ -methionine and immunoprecipitation were conducted by Dr. Frank Cooke.

$5 \times 10^4$  cells were seeded per 50 mm Petri dish in DMEM supplemented with 10% newborn calf serum. After 24 hours interferon ( $10^4$  U/ml) was added to half the dishes. When confluent, cultures were labelled with 250  $\mu\text{Ci}$   $^{35}\text{S}$ -methionine per dish in 0.8 mls Earle's saline for 2 hours and then washed with ice-cold PBS. In cultures grown in the presence of interferon, IFN was also present in the radioactive saline.

Cell lysis, immunoprecipitation and gel electrophoresis

were carried out as described by Shih et al. (227). Anti-serum to p21 was prepared by Scolnick from rats bearing tumours induced by transplanted cells transformed by Harvey Sarcoma Virus (HaSV).

Quantitation of the p21 of each cell type with and without interferon, present in bands revealed by gel electrophoresis was attempted by removing the portion of the gels containing the p21, and then extracting and dissolving the protein in scintillant. The radioactivity (counts per minute) in the p21 were then determined. The ratio of these cpm to three parameters were calculated - total protein content of the cell lysate (determined by Lowry), total cpm of the lysate, and the cpm of three other proteins which were apparently unaltered by interferon treatment.

## RESULTS

### CHAPTER ONE

#### PRODUCTION AND PURIFICATION OF INTERFERON

At the time that this project commenced most studies of interferon activity, both anticellular and antiviral, had been conducted with interferon of specific activity routinely around  $10^6$  to  $10^7$  U/mg of protein. As discussed in the introduction, this raised the possibility that many observations may be due to contaminants and not to interferon at all. Thus, as techniques for purification were improved, so it became essential to routinely use preparations of higher purity.

Initially techniques available in this laboratory were able to yield up to  $2.5 \times 10^7$  units of crude L cell interferon from ten roller bottles, which was then concentrated approximately ten fold by ammonium sulphate precipitation at pH 2. This also purified the interferon roughly ten fold to a specific activity of  $10^6$  U/mg protein, but there were no further purification steps.

Studies with two main aims were thus initiated;

- 1) To maximise the yield of interferon in the crude material, but without increasing the volume if possible.
- 2) To improve the purification procedures so that interferon of close to  $10^8$  units/mg of protein could be routinely produced. This should be achieved without too great a loss of interferon during the purification steps.

#### A) Production of Interferon

Monolayers of L929 Cells The routine procedure followed initially was to induce 10 rollers of confluent L cells with 5mls of NDV per roller (HAU: $10^4$ /ml) for one hour at  $37^{\circ}\text{C}$ , after which the virus suspension was removed and replaced by 25mls of serum-free medium. The interferon-containing medium was harvested 16 hours later. As mentioned above this procedure yielded up to  $2.5 \times 10^7$  units of interferon in 250mls.

Several modifications were introduced to increase this yield.

- 1) Twenty-four hours before induction of confluent L cells, fresh medium was added to each roller bottle to initiate a further round of cell division, thereby increasing cell density to the maximum possible without the cells stripping off the glass due to overcrowding.
- 2) Twenty-five roller bottles were induced instead of ten.
- 3) Only ten millilitres of serum-free medium were added to each roller bottle after induction instead of 25mls.

These three changes ensured that the final volume of the crude remained at 250 mls, but the total interferon yielded rose to about  $2.5 \times 10^9$  units ( $10^8$  units per roller bottle), with a specific activity of up to  $10^7$  U/mg of protein.

Suspension L Cells Induction of suspension L cells was



attempted as a potential method to produce very large quantities of interferon but with less labour than is required with roller bottles. Cultures were maintained in GMEM supplemented with 10% new-born calf serum, at a concentration of  $7 \times 10^5$  to  $1.5 \times 10^6$  cells per ml. Doubling time in agitated cultures was approximately 24 hours. Though these cultures were incubated in a warm room with no  $\text{CO}_2$  buffering, it was found that the cells generated sufficient acid themselves to buffer the medium, provided their density did not fall below  $7 \times 10^5/\text{ml}$  and culture volume was not less than 60% of the total flask volume. It was not possible to continuously grow these cells simply by diluting the cells one to two with fresh medium every day. At least once a week the entire culture had to be sedimented by centrifugation and the cells resuspended in completely fresh medium, suggesting the build-up of a growth inhibitor in the conditioned medium.

For interferon induction the culture size was increased to two litres. Six hours prior to induction the cells were sedimented by centrifugation and resuspended at  $10^6$  cells/ml in fresh medium. For induction the cells were again sedimented and resuspended in a suspension of NDV ( $2 \times 10^4$  HAU/ml) for one hour at  $37^\circ\text{C}$ . This suspension, unlike the cell maintenance cultures, was not stirred. After one hour the cells were sedimented from the virus suspension and resuspended in serum-free medium at  $10^6$  cells/ml. After 16 hours incubation the cells were sedimented once more and the interferon-containing medium harvested.

Titres of approximately  $10^6$  units/ml were obtained, thus yielding  $2 \times 10^9$  units per two litre batch.

Larger yields could easily be increased by inducing a larger culture, the only limitations here being size of flasks and centrifugation facilities available.

However, interferon produced by L cell monolayers was the method eventually chosen for routine production, since although maintenance of a suspension L cell culture was very simple, the number of centrifugation steps and the volumes involved, during the induction process made this procedure very time consuming and put a strain on available centrifugation facilities. Routine production of interferon was taken over by Mr. Robert Bird, and the method he finally adopted after some further studies was that described in Materials and Methods.

All batches of crude interferon were acidified to pH 2 with hydrochloric acid immediately after harvesting and stored at  $4^{\circ}\text{C}$  for three to five days before purification by the methods described below.

#### B) Purification

The strategy adopted in studies of purification was to test the effectiveness of several methods already published in the literature and adapt the most successful technique to our own uses to give a product with specific activity of about  $10^8$  units/mg of protein.

Carter and his colleagues had already tested a number of affinity adsorbents for their potential in affinity chromatographic purification of interferon (287, 288), and so two of their most successful adsorbents were tested - BSA-Sepharose 4B, and Affi-gel 202.

BSA-Sepharose 4B Huang et al. (288) first demonstrated the use of this adsorbent to purify human fibroblast interferon, binding the interferon in phosphate buffer at pH 7.2 and releasing it with ethylene glycol, but were unable to purify mouse interferon by this method. Davey et al. (287) modified the technique to bind interferon in acetate at pH 5 and so purify  $4 \times 10^4$  units of crude L cell interferon from  $10^5$  U/mg of protein to  $10^8$  U/mg protein in a single step with 90% recovery. The method they employed was as follows:

Crude interferon was dialysed for 24 hours against 0.05 M sodium acetate, pH 5.0, and then applied to the BSA-Sepharose column which was equilibrated with the same buffer. The pH of the column was then raised to 7.4 by washing extensively with 0.02 M disodium monohydrogenphosphate. Interferon was then eluted by a sodium chloride gradient of 0 to 0.5 M in the 0.02 M phosphate buffer, with the interferon eluting in the early part of the gradient.

The method adopted in our studies was essentially the same except that ammonium sulphate-precipitated interferon was used instead of crude, and a much higher titre was applied

to the column - a total of  $2 \times 10^6$  units. Furthermore a sodium chloride gradient was not applied to elute the interferon, just a fixed concentration of either 1.0 M or 0.1 M NaCl dissolved in the buffer.

Figure one and Table one show the results of the first experiment, in which 1.0 M NaCl was used to elute the interferon. As can be seen, the vast bulk of the protein ran straight through in the acetate breakthrough, but this only contained 0.05% of the interferon. Washing with phosphate buffer at pH 7.4 released approximately 1% of the interferon. These results were in accord with those observed by Davey et al. but the results of the salt elution were rather disappointing. Only 50% of the interferon was released and the highest specific activity attained showed just a four fold purification of the starting material.

It seemed possible that use of a lower salt concentration might release less non-interferon protein bound strongly to the BSA, so the experiment was repeated, but this time the interferon was eluted initially by 0.1 M NaCl and then followed by 1.0 M NaCl.

Figure 2 and Table 2 illustrate that interferon purity was indeed improved by eluting with a lower salt concentration. 0.1 M NaCl eluted the interferon, though with only 30% yield, the highest specific activity attained being  $10^7$  U/mg protein, while 1.0 M NaCl released no more interferon but doubled the release of protein.

Fraction	Volume (mls)	Total Interferon (Units)	Total Protein (mg)	Specific Activity (U/mg)
B' through 1	10	794	0.67	$1.2 \times 10^3$
B' through 2	10	214	0.11	$2.0 \times 10^3$
Washings	10	$1.4 \times 10^4$	0.14	$1.0 \times 10^4$
Salt 1	10	$1 \times 10^6$	0.24	$4.8 \times 10^6$
Salt 2	10	$1.6 \times 10^4$	0.14	$1.1 \times 10^5$
Salt 3	10	$2.5 \times 10^4$	0.14	$1.8 \times 10^5$

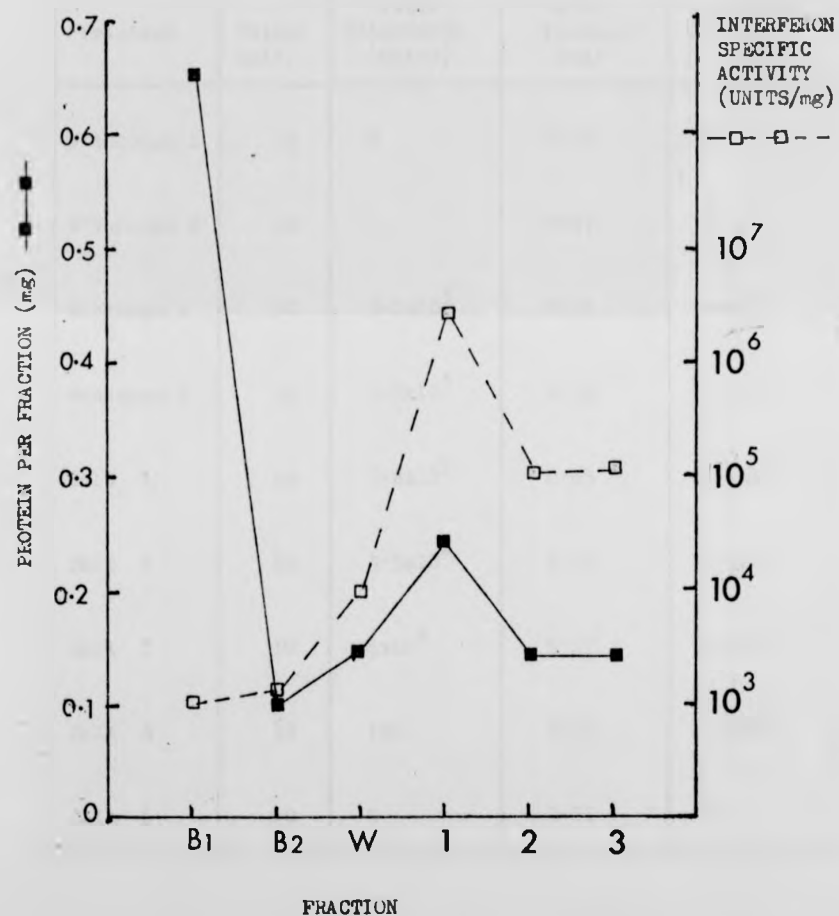
TABLE 1    Purification of Interferon by Affinity Chromatography  
on BSA-Sepharose 4B (Experiment 1)

KEY TO TABLES 1 TO 4

B - BREAKTHROUGH

W - PHOSPHATE BUFFER WASHINGS

1 to 5 - SALT ELUATE FRACTIONS



**FIGURE 1** Purification of Interferon by Affinity  
Chromatography on BSA-Sepharose 4B (Experiment 1)

KEY TO FIGURES 1 TO 4

BREAKTHROUGH - B

PHOSPHATE WASHINGS - W

SALT WASHINGS - 1 to 5

Fraction	Volume (mls)	Total Interferon (Units)	Total Protein (mg)	Specific Activity (U/mg)
B'through 1	10	0	0.59	0
B'through 2	10	0	0.07	0
Washings 1	10	$3.2 \times 10^3$	0.11	$3 \times 10^4$
Washings 2	10	$1.0 \times 10^3$	0.39	$2.6 \times 10^3$
Salt 1	10	$5.6 \times 10^5$	0.05	$1.1 \times 10^7$
Salt 2	10	$3.5 \times 10^4$	0.05	$7.0 \times 10^5$
Salt 3	10	$5 \times 10^4$	0.07	$7.1 \times 10^5$
Salt 4	10	100	0.07	$1.4 \times 10^3$
Salt 5	10	0	0.11	0

TABLE 2    Purification of Interferon by Affinity Chromatography  
on BSA-Sepharose 4B (Experiment 2)

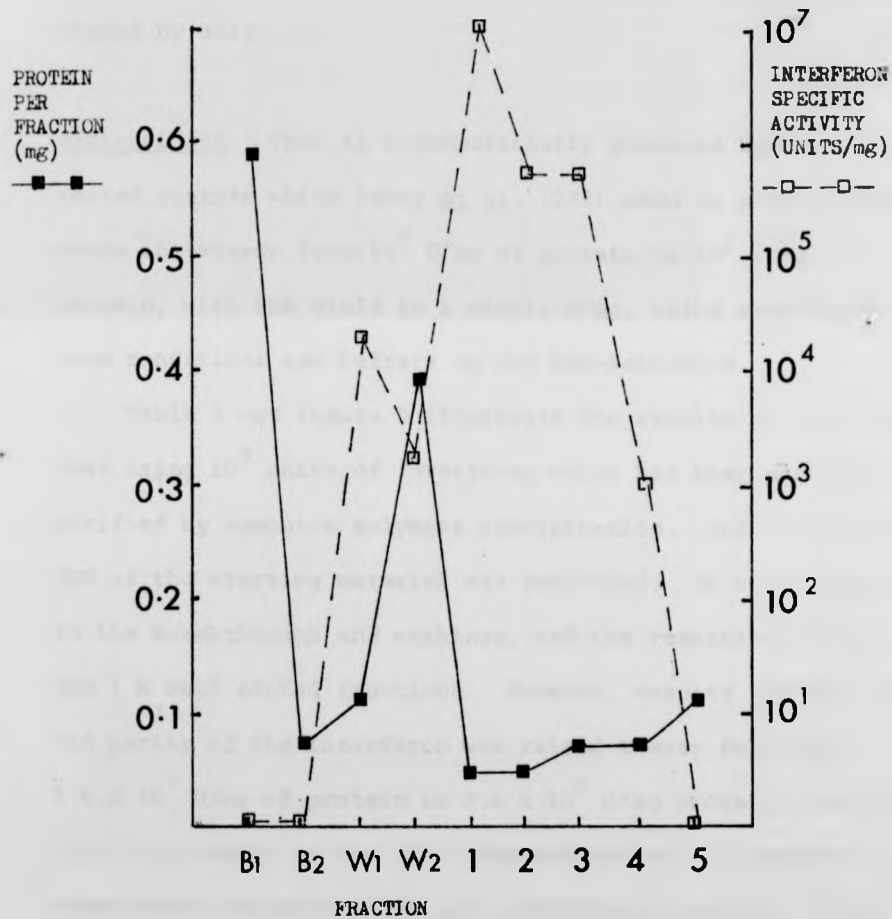


FIGURE 2 Purification of Interferon by Affinity  
Chromatography on BSA-Sepharose 4B (Experiment 2)



Subsequent experiments with crude interferon were less successful. In one typical experiment 100% of the interferon bound, but 5% was released in the washings and only 10% was eluted by salt.

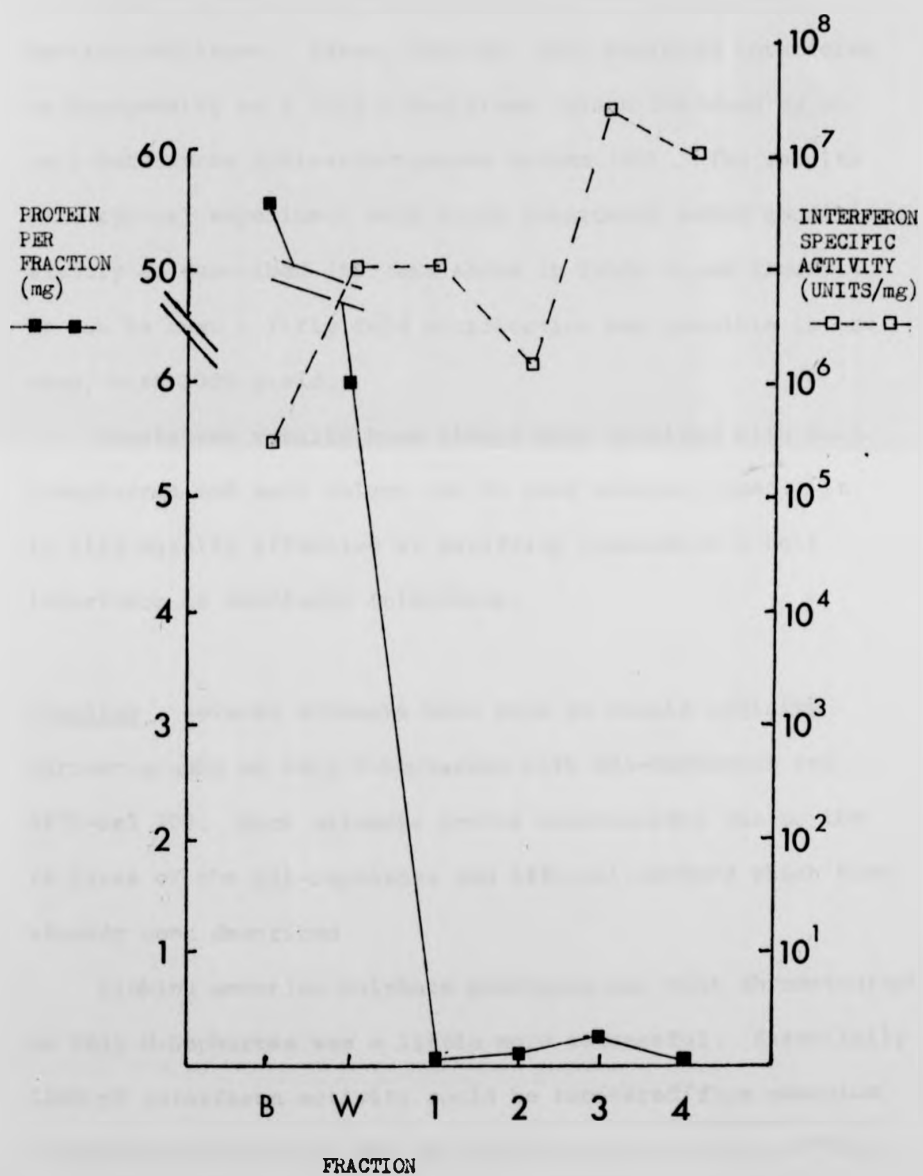
Affi-gel 202 This is a commercially produced hydrocarbon-coated agarose which Davey et al. (287) used to purify crude mouse interferon from  $10^5$  U/mg of protein to  $10^8$  U/mg of protein, with 95% yield in a single step, using exactly the same conditions and buffers as for BSA-Sepharose.

Table 3 and figure 3 illustrate the results of the first test using  $10^9$  units of interferon which had been partially purified by ammonium sulphate precipitation. Only a total of 20% of the starting material was recovered, 9% of it being in the breakthrough and washings, and the remaining 11% in the 1 M NaCl eluted fractions. However, despite the low yield, the purity of the interferon was raised twenty fold from  $1.6 \times 10^7$  U/mg of protein to  $3.8 \times 10^8$  U/mg protein, a considerable improvement on the BSA-Sepharose method. Subsequent experiments failed to show such encouraging results. Close to 100% of the interferon bound to the column, but NaCl failed to elute any. Application of up to 50% ethylene glycol also failed to remove the interferon.

Poly U-Sepharose De Maeyer-Guignard et al. (57) first observed that mouse interferon could bind to polynucleotides,

Fraction	Volume (mls)	Total Interferon (Units)	Total Protein (mg)	Specific Activity (U/mg)
Starting IFN	40	$1.1 \times 10^9$	70	$1.6 \times 10^7$
B'through	40	$2.5 \times 10^7$	55.6	$4.5 \times 10^5$
Washings	30	$6.6 \times 10^7$	6.0	$1.1 \times 10^7$
Salt wash 1	2	$5 \times 10^5$	0.06	$1.0 \times 10^7$
" " 2	2	$2.0 \times 10^5$	0.1	$2.0 \times 10^6$
" " 3	2	$1.0 \times 10^8$	0.26	$3.8 \times 10^8$
" " 4	2	$3.6 \times 10^6$	0.032	$1.1 \times 10^8$

TABLE 3 Purification of Interferon by Affinity Chromatography  
on Affi-Gel 202



**FIGURE 3** Purification of Interferon by Affinity Chromatography  
on Affi-Gel 202

and this was used as a method to elute interferon from Blue Dextran-Sepharose. Later, however, they purified interferon to homogeneity on a Poly U-Sepharose column followed by an anti-interferon antiserum-agarose column (58). The results of a typical experiment with crude interferon using conditions exactly as described (58) are shown in Table 4 and figure 4. As can be seen a fifty fold purification was possible in one step, with 100% yield.

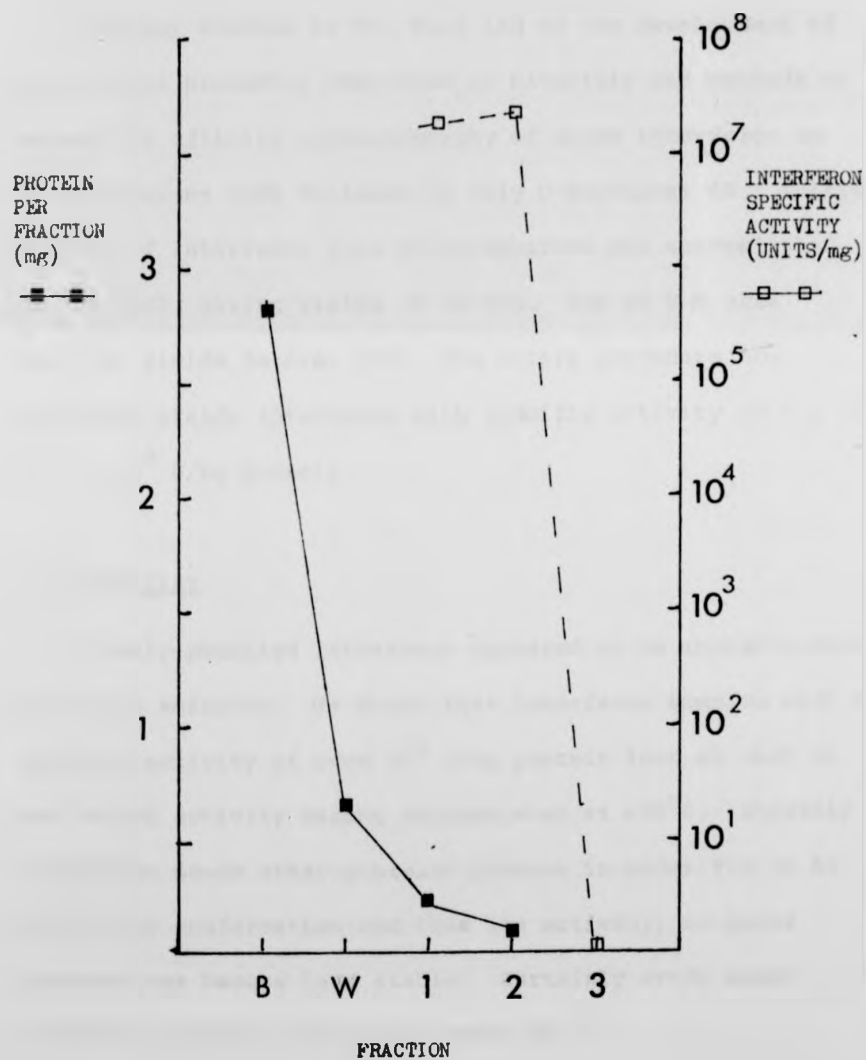
Consistent results have always been obtained with Poly U-Sepharose and each column can be used several times. It is also equally effective at purifying suspension L cell interferon as monolayer interferon.

Coupling Several attempts were made to couple affinity chromatography on Poly U-Sepharose with BSA-Sepharose and Affi-Gel 202. Such attempts proved unsuccessful due to the failures of the BSA-Sepharose and Affi-gel methods which have already been described.

Linking ammonium sulphate precipitation with chromatography on Poly U-Sepharose was a little more successful. Essentially 100% of interferon activity could be recovered from ammonium sulphate precipitation yet up to 90% (or one log) of activity was often lost, especially when large interferon samples were used (more than  $10^9$  units) between the precipitation and the chromatography. This presumably took place during dialysis of the interferon into 10mM Tris-HCl for the chroma-

Fraction	Volume (mls)	Total Interferon (Units)	Total Protein (mg)	Specific Activity (U/mg)
Starting IFN	50	$1.25 \times 10^7$	25.0	$5.0 \times 10^5$
Salt Wash 1	2	$8.0 \times 10^6$	0.36	$2.2 \times 10^7$
" " 2	2	$4.4 \times 10^6$	0.16	$2.75 \times 10^7$

TABLE 4 Purification of Interferon by Affinity Chromatography  
on Poly U-Sepharose 4B



**FIGURE 4** Purification of Interferon by Affinity Chromatography  
on Poly U-Sepharose 4B

tography. This was observed repeatedly with interferon from both monolayer and suspension L cells by both myself and Mr. Robert Bird.

Further studies by Mr. Bird led to the development of the routine procedure described in Materials and Methods of sequential affinity chromatography of crude interferon on Blue-Sepharose CL6B followed by Poly U-Sepharose 4B. Initially, elution of interferon from Blue-Sepharose was accomplished by 1 M NaCl, giving yields of 30-70%. Use of 8 M urea improved yields to over 70%. The entire procedure now routinely yields interferon with specific activity of  $5 \times 10^7$  to  $2 \times 10^8$  U/mg protein.

#### C) Stability

Highly purified interferon appeared to be unstable when in dilute solution. We found that interferon samples with a specific activity of over  $10^8$  U/mg protein lost as much as one log of activity during storage, even at  $-70^\circ\text{C}$ . Possibly interferon needs other proteins present in order for it to retain its conformation and thus its activity, so purer preparations become less stable. Certainly crude mouse interferon remains stable for weeks at  $4^\circ\text{C}$ .

An initial attempt to overcome this was made by adding BSA at concentrations up to 3 mg/ml to purified samples, which appeared to be at least partially effective. Later batches, purified by Blue-Sepharose and Poly U-Sepharose, were

found to be quite stable if kept in DMEM supplemented with 10% new-born calf serum and stored in liquid nitrogen.

#### D) Discussion

Huang et al. (288) found it was necessary to use ethylene glycol treatment to release human interferon bound to BSA-Sepharose at neutral pH, thus suggesting that the interferon was hydrophobically bound. Davey et al. (287), however, were able to bind mouse interferon to BSA at pH 5 and release it at pH 7.2 with salt only. This suggested that electrostatic interactions may be more important in interferon binding.

If hydrophobic interactions were playing a major role in our experiments, then the use of ethylene glycol may have released the bound interferon. However, hydrophobic bonding is almost certainly very important with Affi-gel 202, yet ethylene glycol failed to release bound interferon.

Binding of interferon to polynucleotides in general and poly U in particular is probably electrostatic in nature, being easily reversed by the addition of salt. The physiological significance, if any, of such a specific binding as this is still unknown. The fact that double-stranded polynucleotides are inducers of interferon both in tissue culture and in animals has led some to suggest that the polynucleotide-interferon binding is important in the induction of interferon (236).

At the start of this study experiments by most workers



routinely used interferon of specific activity around  $10^6$ - $10^7$  units/mg protein, though several laboratories had claimed to have purified various interferons to homogeneity. Knight (144) in 1975 purified mouse interferon by a four step procedure to give a preparation containing 10-11 polypeptides, apparently all with interferon activity, with total specific activity of  $2.5 \times 10^8$  U/mg protein. Subsequent studies since 1978 (45, 58, 134, 141, 245) have purified mouse interferon to more than  $10^9$  U/mg protein containing only two active polypeptides. All such preparations possess both antiviral and anticellular activities.

The drawbacks with most of these techniques which purify interferon to homogeneity are that most have several steps. This makes the process extremely time-consuming and gives very low yields at the finish. For instance, Iwakura *et al.* (134) purified interferon from mouse L cells to a specific activity of  $2.6 \times 10^9$  units/mg protein in a process that used eight steps but gave a yield of less than 1% of the starting material. Similarly, with purification of human alpha and beta interferons, multistep procedures have purified interferon to homogeneity but have often only obtained low yields (24, 145, 216, 249, 284, 285).

Due to the problems of obtaining large quantities of pure interferon the majority of studies on interferon's activities are still conducted with material of specific activity in the range of  $10^7$ - $10^8$  units/mg protein since large

quantities of interferon of this purity can be routinely achieved. A few purification techniques have, however, been developed which give highly purified interferon in high yields, e.g. the two-step procedure developed by de Maeyer-Guignard et al. (58) for mouse interferon using Poly U-Sepharose and anti-interferon antiserum, and purification of human fibroblast interferon by Knight et al. (147) by Blue-Sepharose and polyacrylamide gel electrophoresis, which suggests that it may soon become possible to routinely employ pure interferon in many more studies.

Using interferon purified by Poly U-Sepharose and antibody agarose, De Maeyer-Guignard and her colleagues have recently published the results of studies employing homogeneous mouse interferon (58, 59, 82).

In summary, the results presented in this chapter show the efficiency of various agents in acting as affinity adsorbents for the purification of mouse interferon and described the development of a technique which can routinely achieve specific activities of  $5 \times 10^7$  to  $2 \times 10^8$  units/mg protein with at least a 70% recovery of the initial crude material. This procedure, whilst obtaining interferon which is at best 10% pure, compares favourably with methods used by others to produce interferon used routinely in studies of interferon's antiviral and anticellular activities.

## CHAPTER TWO

### EFFECTS OF INTERFERON ON GROWTH OF CELLS

On a number of occasions interferon has been reported to inhibit the growth of both normal and transformed cells, and several studies (86, 143, 157, 166, 244) have found that interferon may affect the two types of cells differentially.

The series of experiments described in this chapter studied the effect of interferon on various parameters of cell growth in an attempt to establish if differences between the responses of normal and transformed cells to interferon do exist.

#### A) Sensitivities to Antiviral and Cell Growth Inhibitory Activities

Inhibition of SFV replication (as measured by  $^3\text{H}$ -uridine incorporation in AMD-treated cells) and cellular DNA synthesis (as measured by  $^3\text{H}$ -thymidine incorporation) by a range of interferon doses ( $10^4$  to  $10^{-3}$  units/ml) in several cell lines is shown in the dose response curves of figures 5 and 6. The data presented in Table 5 show that the sensitivities to interferon's antiviral activity vary widely, a range of about 1000 fold being seen in just these eight cell lines. By contrast, sensitivities to cell growth inhibition are remarkably similar, varying by only 15 fold. However, since these cells were incubated for three days in the presence of interferon, before labelling with  $^3\text{H}$ -thymidine, the

# EXPERIMENTAL STUDY OF THE EFFECT OF TEMPERATURE ON THE RATE OF REACTION OF HYDROGEN PEROXIDE WITH FERROUS SULFATE



LEGEND TO FIGURE 5

<sup>3</sup>H-Uridine incorporation measures only viral replication.

Cellular RNA synthesis is inhibited by the presence of actinomycin D.

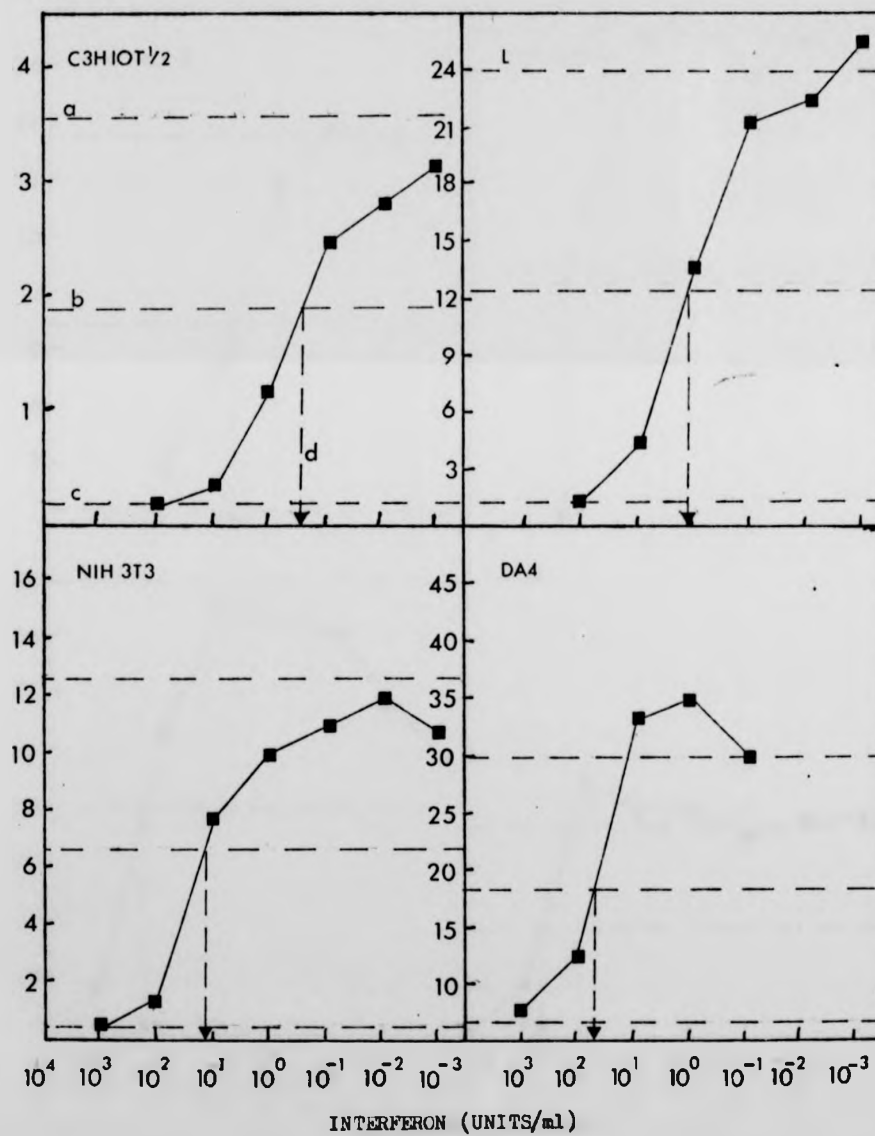
Dotted lines represent the following -

- a - 100% virus replication (virus control)
- b - 50% virus replication
- c - 0 virus replication (cell control)
- d - Interferon dose that would reduce viral replication to 50% of virus control (VI50).

This experiment was conducted by the same method as that employed for routine interferon assays (286). Basically, cells were seeded in glass vials (area of base 1cm<sup>2</sup>) at 10<sup>5</sup> cells per vial, and incubated for 24 hours in 1ml of DMEM supplemented with 10% new-born calf serum. At the end of this period the medium was removed and replaced with 1ml medium containing 2% new-born calf serum and a range of doses of interferon. After a further 24 hour incubation this medium was replaced by 0.25ml DMEM plus 2% new-born calf serum containing actinomycin D (1µg/ml) and Semliki Forest Virus (SFV). The AMD inhibits the cellular DNA-directed RNA synthesis, but not the viral RNA-directed RNA synthesis. After a 3 hour incubation 0.25 ml DMEM containing 2% new-born calf serum, AMD and <sup>3</sup>H-uridine (1µCi/ml) was added to each vial for a further 2½ hours. Incorporation of <sup>3</sup>H-uridine into the acid precipitable material was then recorded in order to measure SFV replication.

FIGURE 5

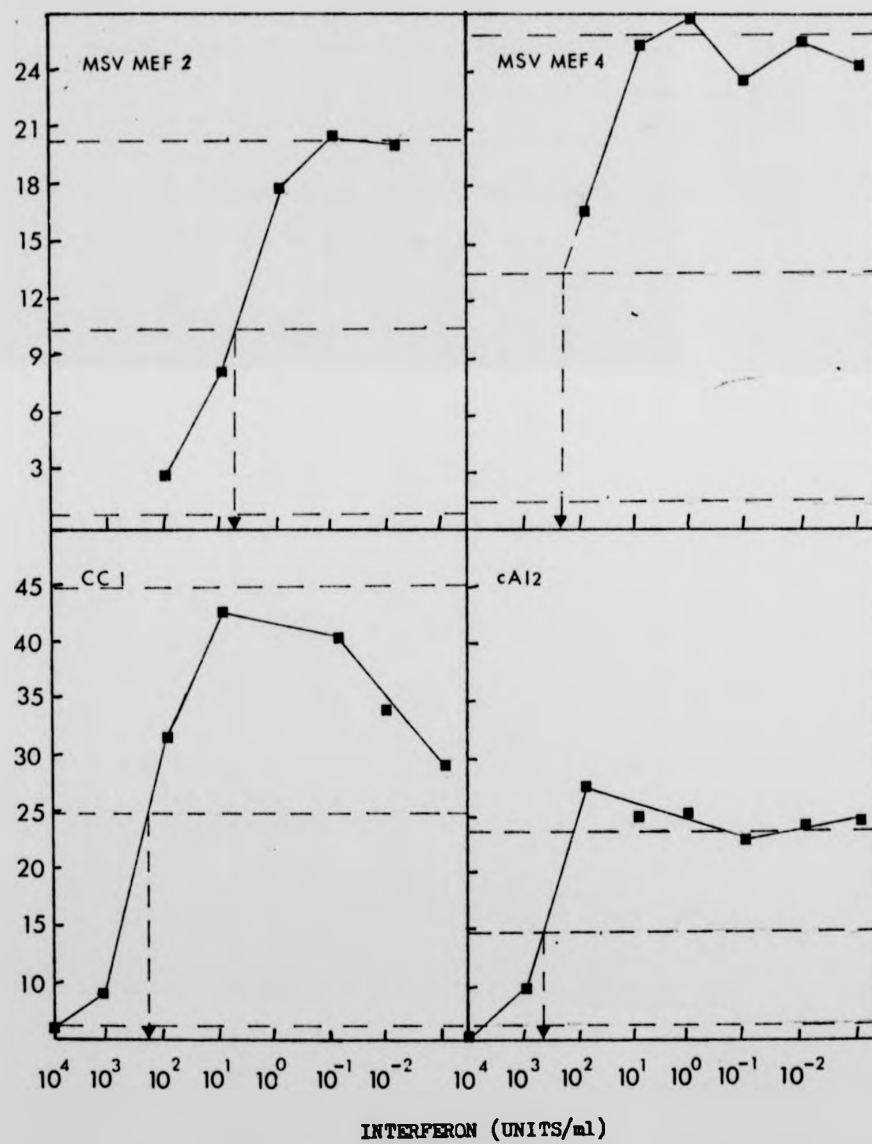
$^3\text{H}$ -URIDINE  
CPM  $\times 10^{-3}$



Sensitivity of Cell Lines to Interferon's Antiviral Activity

FIGURE 5 cont/d.

<sup>3</sup>H-URIDINE  
CPM x 10<sup>-3</sup>



LEGEND TO FIGURE 6

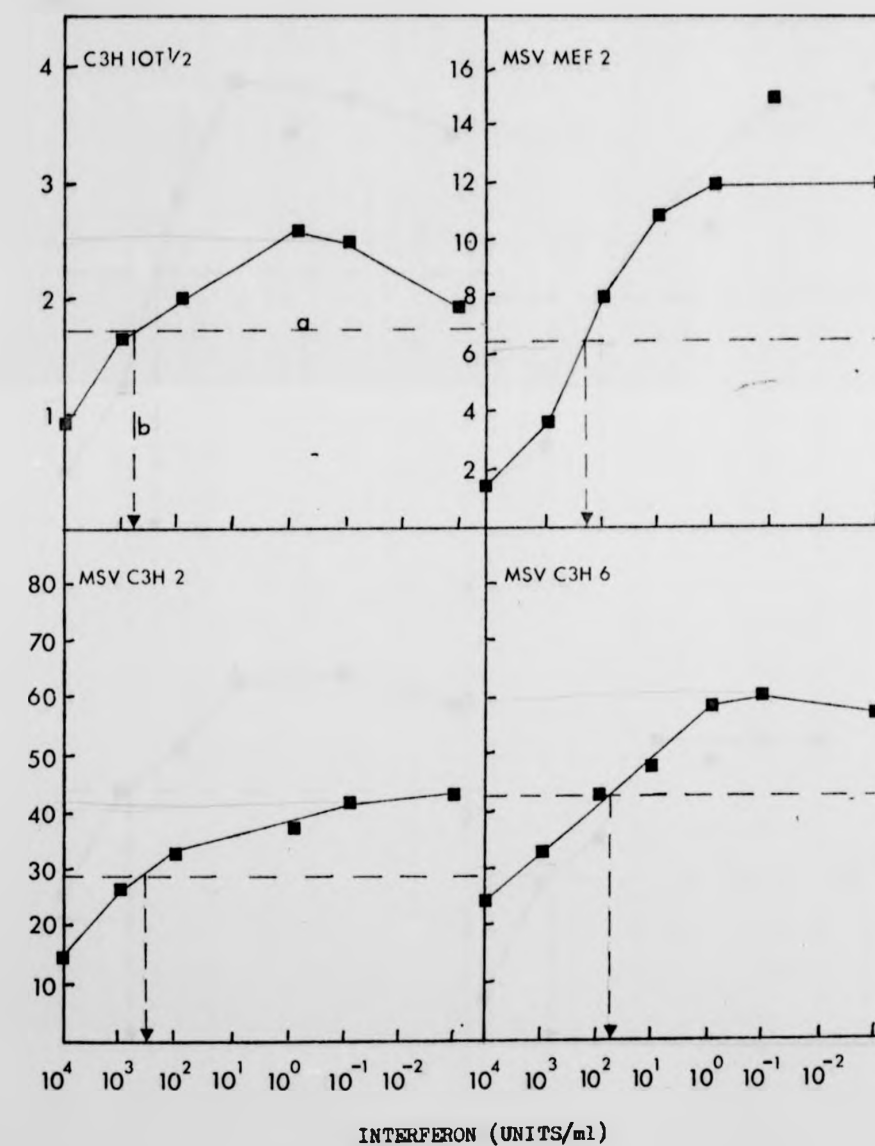
$^3\text{H}$ -Thymidine incorporation measured the level of DNA synthesis in the cultures.

Dotted lines represent the following -

- a - 50% inhibition of cellular DNA synthesis.
- b - Interferon dose causing 50% inhibition of cellular DNA synthesis.

$^3\text{H}$ -THYMIDINE  
CPM  $\times 10^{-3}$

FIGURE 6



Sensitivity of Cell Lines to the Growth Inhibitory  
Activity of Interferon



FIGURE 6 cont/d.

<sup>3</sup>H-THYMIDINE  
CPM x 10<sup>-3</sup>

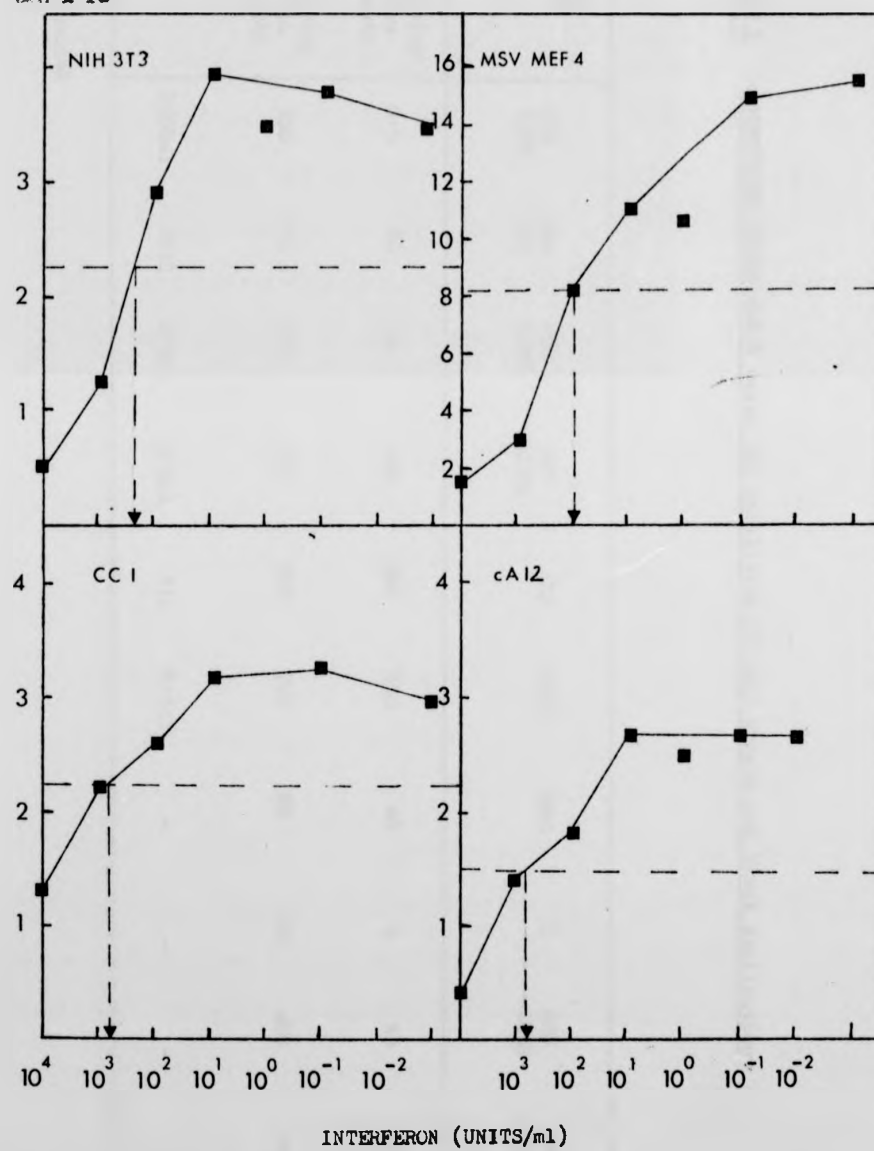


TABLE 5

Interferon Doses which cause 50% Inhibition of Cell Growth and Virus Replication

CELL LINE EXPT.	C3H 10T $\frac{1}{2}$	NIH 3T3	MSV MEF2	MSV MEF4	CC1	CA12	DA4	L	MSV C3H2	MSV C3H6
IFN(U/ml) giving 50% inhibition of virus growth (VI50)	0.5	20	70	200	300	550	60	1	ND	ND
IFN(U/ml) giving 50% inhibition of cell growth (GI50)	500	400	200	100	900	700	ND	ND	400	60
RATIO OF GI50/VI50	1000:1	20:1	2.9:1	0.5:1	3:1	1.3:1	-	-	-	-

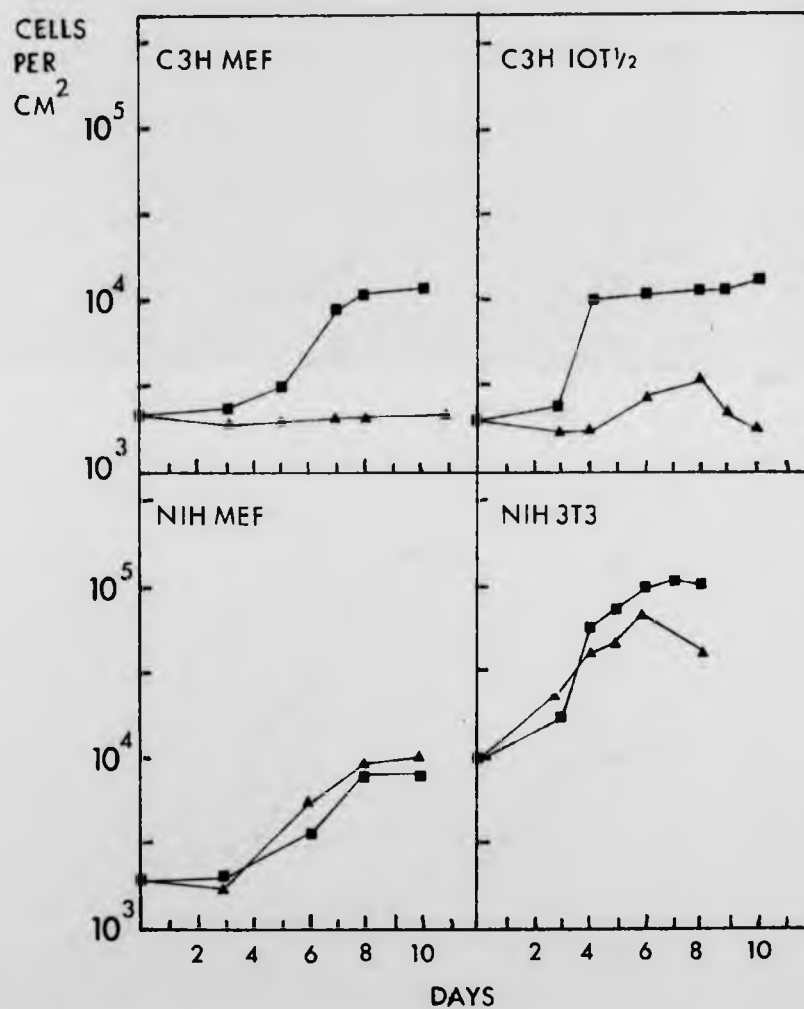
ND - Not Determined

observed reduction in  $^3\text{H}$ -thymidine incorporation at high interferon doses was due not only to a slower rate of DNA synthesis, but also to lower cell numbers. The dose-response curves were, therefore difficult to interpret. A 24 hour interferon treatment before labelling with  $^3\text{H}$ -thymidine would have avoided the complication of reduced cell numbers so that reduced rate of DNA synthesis only was measured. Such data are presented in paragraph C of this chapter, though only with one interferon dose. These results will be compared in the Discussion.

The maximum effect on DNA synthesis in these experiments was observed at an interferon dose of  $10^4$  units/ml, so this dose was used in most subsequent experiments.

#### B) Growth and Saturation Densities

Growth curves were determined with and without  $10^4$  units/ml of interferon for various cell lines (fig. 7). C3H10T $\frac{1}{2}$ , NIH MEF and C3H MEF cells without interferon showed the growth characteristic typical of normal cells whose growth is subject to density dependent inhibition, i.e. rapid initial growth to a saturation density of  $1-3 \times 10^4$  cells per  $\text{cm}^2$ . In the presence of interferon the growth of C3H10T $\frac{1}{2}$  and C3H MEF cells was very severely inhibited, but growth of NIH MEF cells was unaffected. In the absence of interferon, the MSV transformants of the C3H10T $\frac{1}{2}$  and NIH MEF cells grew initially at a rate very similar to normal cells, but they reached

FIGURE 7

Effects of Interferon on Growth and Saturation Densities  
of Several Cell Lines

—■— 0 IFN  
 —▲— 10<sup>4</sup> UNITS/ml IFN

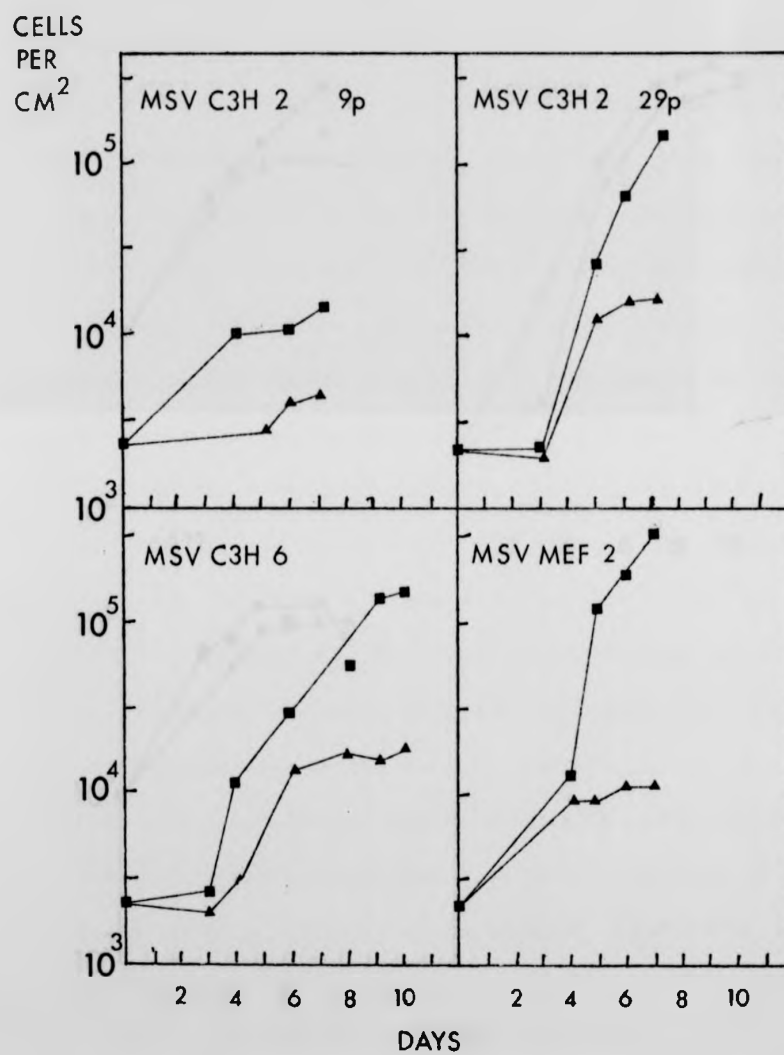
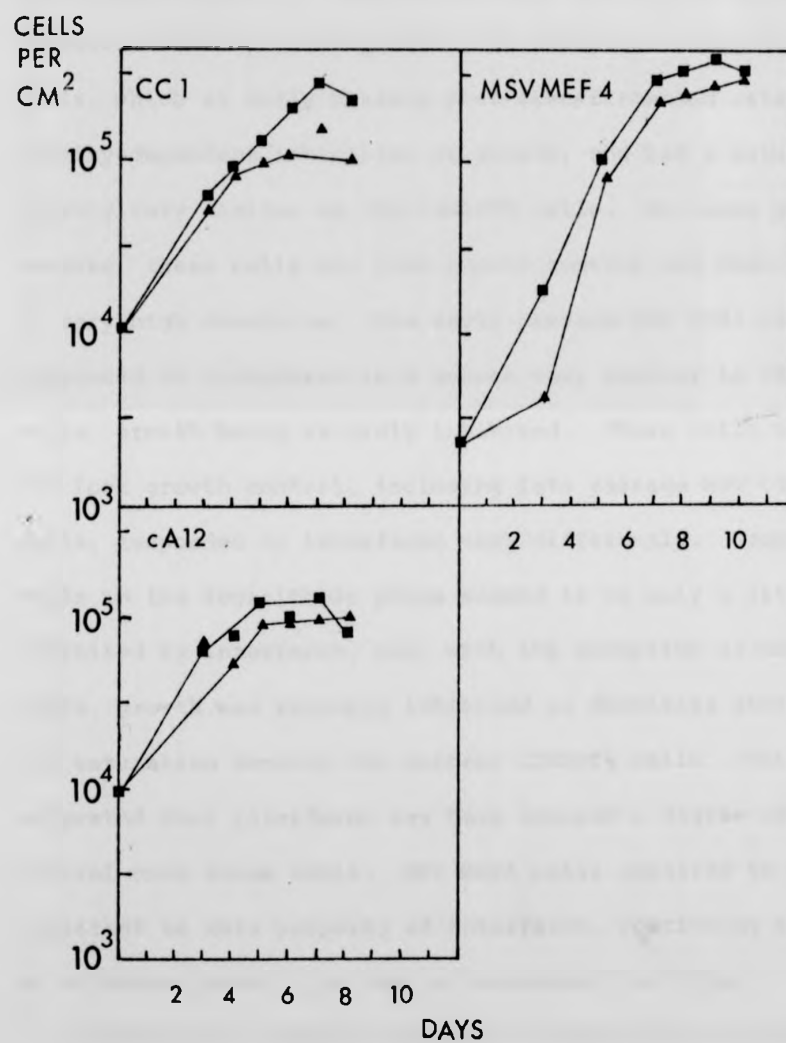
FIGURE 7 cont/d.

FIGURE 7 cont/d.

densities ten to twenty times greater than their non-transformed parents. These cells had, thus, lost density-dependent inhibition of growth. An exception were MSV C3H2 cells, which at early passage post-transformation retained density-dependent inhibition of growth, and had a saturation density very similar to the C3H10T $\frac{1}{2}$  cells. At later passages, however, these cells too lost growth control and then grew to very high densities. The early passage MSV C3H2 cells responded to interferon in a manner very similar to C3H10T $\frac{1}{2}$  cells, growth being severely inhibited. Those cells that had lost growth control, including late passage MSV C3H2 cells, responded to interferon very differently. Growth of cells in the logarithmic phase seemed to be only a little inhibited by interferon, but, with the exception of MSV MEF4 cells, growth was strongly inhibited at densities similar to the saturation density for control C3H10T $\frac{1}{2}$  cells. This suggested that interferon may have imposed a degree of growth control onto these cells. MSV MEF4 cells appeared to be resistant to this property of interferon, continuing to grow to a density similar to that of untreated cultures.

There was a possibility that the cessation of growth by MSV C3H2 and 6 and MSV MEF2 at a low cell density may be due to a delayed toxic effect of interferon, in which case if interferon-treated cells were reseeded at very low density, still in the presence of interferon, no new growth should occur. This was tested with MSV MEF2 cells by repeatedly

passing the cells in the presence of interferon. At each passage the untreated controls grew to a density 5 to 10 fold higher than that of the interferon-treated cultures (15 to  $20 \times 10^6$  versus 2 to  $4 \times 10^6$  cells per  $25\text{cm}^2$  flask), thus demonstrating that a delayed cytotoxicity is not the cause of the growth inhibition. Similar results with a wide range of other cell lines have been obtained by Dr. A. Morris.

The NIH 3T3 4E cells and their transformants, CCl and cAl2, appeared to be less sensitive to interferon's activity. The nontransformed NIH 3T3 4E cells grew to almost as high a density as the CCl and cAl2, but interferon treatment did reduce it somewhat, as well as reducing the growth rate. Interferon reduced the growth rate of the cAl2 cells but not the CCl cells, and conversely the saturation density of CCl cells was reduced, but that of cAl2 was barely affected. Interestingly, in interferon-treated cultures of NIH 3T3, CCl and cAl2 cells the saturation densities were all very similar - about  $10^5$  cells per  $\text{cm}^2$ .

Despite the fact that both C3H10T $\frac{1}{2}$  cl8 and NIH 3T3 4E cells were used in these experiments at early post-cloning passages (less than 15 passages), it is clear that they behaved very differently, the C3H10T $\frac{1}{2}$  cl8 cells being much more tightly growth controlled and growth rate being much more severely hindered by interferon. The NIH 3T3 cells had a tendency to undergo spontaneous transformation after about 20 passages in culture, and even at the earliest passages the



cells were smaller and the microfilament stress fibre system was less extensive than in early passage C3H10T $\frac{1}{2}$  cl8 cells, in addition to the higher saturation density (results not shown). It is quite possible that this state of partial transformation may have been partly responsible for the different behaviour of these cells.

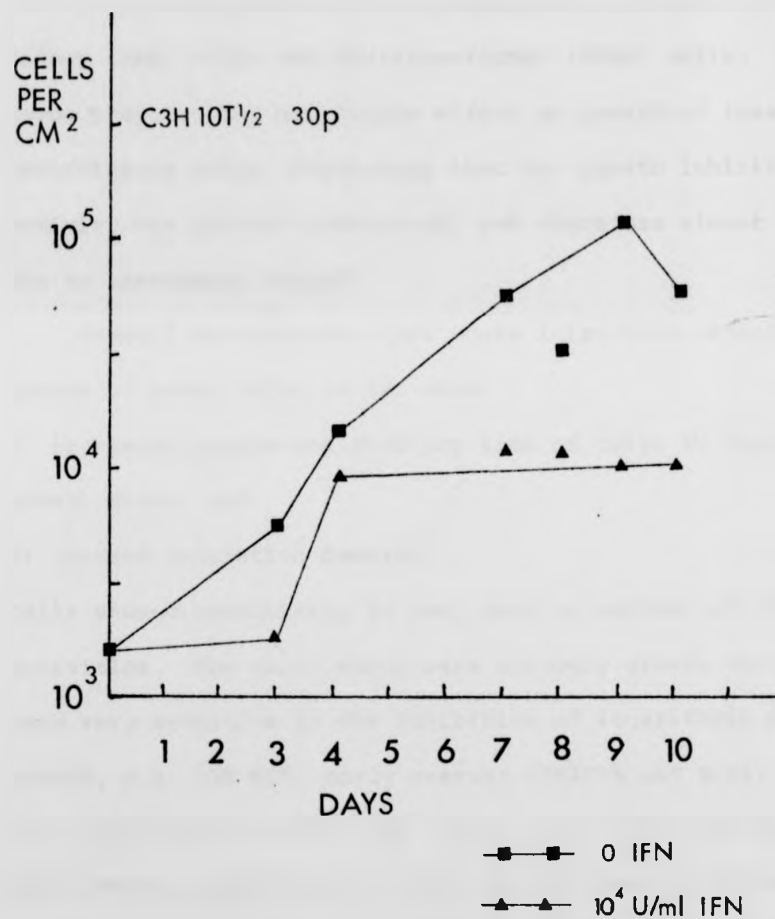
When growth curves for C3H 10T $\frac{1}{2}$  cl8 cells at later passage (passage 30) were measured, the results were very different from those obtained with early passage cells (figure 8). These cells showed at least a partial loss of density-dependent inhibition of growth, and reached a saturation density several fold higher than that observed for early passage C3H10T $\frac{1}{2}$  cl8 cells. In addition cell size was also reduced. This would suggest that these cells may be undergoing progressive spontaneous transformation. The response of these cells to interferon treatment was also very similar to that seen with the density-independent MSV-transformed cells. The logarithmic growth phase was only inhibited a little by interferon, but growth was strongly inhibited at a density very similar to early passage C3H10T $\frac{1}{2}$  cl8 cells - about  $2 \times 10^4$  cells/cm<sup>2</sup>. These data, plus those obtained with the MSV C3H2 cells, suggest that the way in which MSV-transformed cells respond to interferon is not due to MSV transformation per se, but is determined more by the degree of growth control shown by a cell type.

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FIGURE 8

Effect of Interferon on Growth of Late Passage (30th passage)

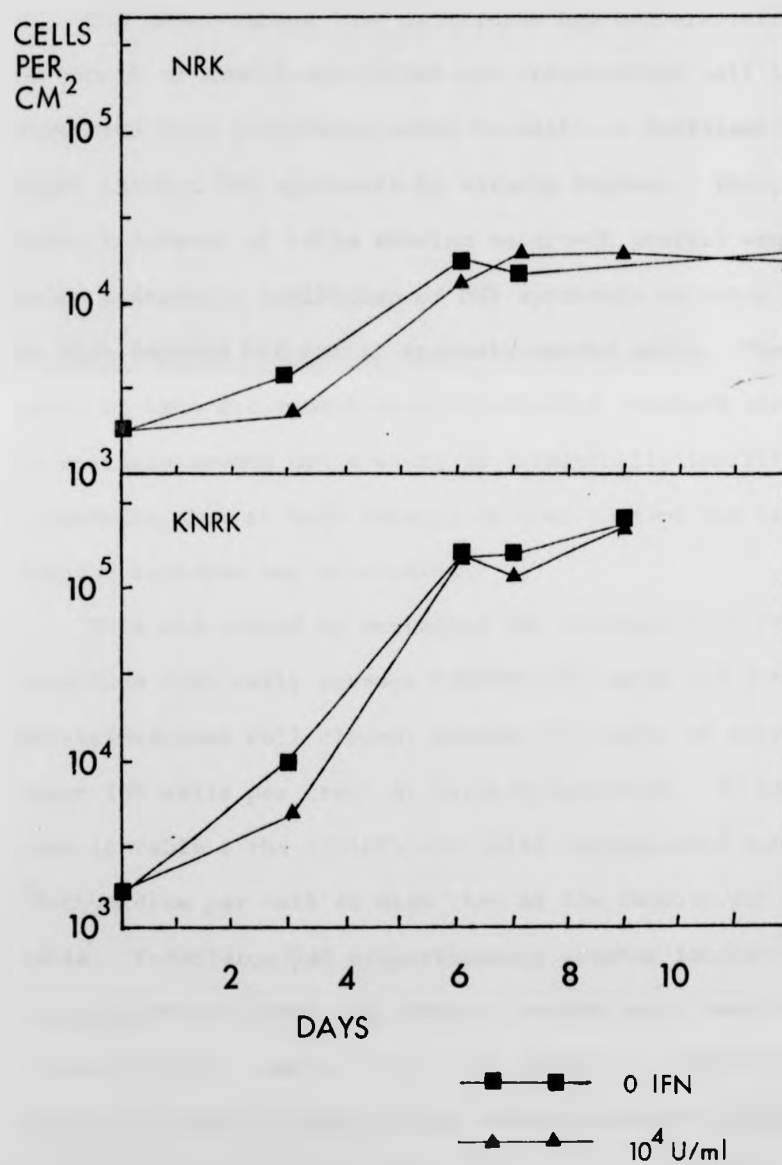
C3H 10T<sup>1/2</sup> Cells.

preparations used, some unknown contaminant may be responsible for the growth effects observed. Several preparations were therefore tested on heterologous rat cells. Figure 9 shows the results obtained with one preparation on Normal Rat Kidney (NRK) cells and MSV-transformed (KNRK) cells. Interferon preparations had little effect on growth of these heterologous cells, confirming that the growth inhibitory activity was species restricted, and therefore almost certainly due to interferon itself.

Overall the data show that mouse interferon affected the growth of mouse cells in two ways:

- a) increased population doubling time of cells in logarithmic growth phase, and
- b) lowered saturation density.

Cells showed sensitivity to one, both or neither of these activities. The cells which were strongly growth controlled were very sensitive to the inhibition of logarithmic phase growth, e.g. C3H MEF, early passage C3H10T $\frac{1}{2}$  and early passage post-transformation MSV C3H2, while those which had lost, or were losing, growth control were usually less sensitive to the effect of interferon on logarithmic phase growth, but showed reduced saturation densities, e.g. MSV C3H6, MSV MEF2, late passage post-transformation MSV C3H2, NIH 3T3 and late passage C3H10T $\frac{1}{2}$ . Other cell types were insensitive to both effects, e.g. NIH MEF and MSV MEF4.

FIGURE 9

Lack of Effect of Mouse Interferon on Growth of  
Heterologous (Rat) Cells.

C) Effects of Cell Density on Interferon's Inhibition of DNA Synthesis

The observations that interferon had differential effects on growth of growth-controlled and uncontrolled cell lines suggested that interferon added to cells at different densities might inhibit DNA synthesis by varying degrees. Thus, interferon treatment of cells showing no growth control would cause a dramatic inhibition of DNA synthesis in cells seeded at high density but not in sparsely-seeded cells. The reverse would be true for growth controlled cells in which growth of sparsely-seeded cells would be dramatically inhibited by interferon, but at high density neither control nor interferon-treated cultures may be growing.

This was tested by measuring the incorporation of  $^3\text{H}$ -thymidine into early passage C3H10T $\frac{1}{2}$  cl8 cells and three MSV-transformed cell clones, seeded into wells of microtitre trays (96 wells per tray) at varying densities. As can be seen in Table 6 the C3H10T $\frac{1}{2}$  cl8 cells incorporated much less  $^3\text{H}$ -thymidine per cell at high than at low density for these cells. Interferon had proportionally greater inhibitory activity at the lower cell density, where rapid growth would normally occur, than at the higher densities where control cells had ceased growing due to density dependent growth inhibition.

The transformed cells, in the absence of interferon, incorporated roughly the same amount of radioactive thymidine

TABLE 6

CELL LINE	IFN U/ml	Incorporation of $^3\text{H}$ -Thymidine into acid-insoluble Material mean cpm per culture $\times 10^{-3}$			
		No. of cells per well			
		500 (1000/cm <sup>2</sup> )	2000 (4000/cm <sup>2</sup> )	10000 (20000/cm <sup>2</sup> )	20000 (40000/cm <sup>2</sup> )
C3H 10T $\frac{1}{2}$	0	2.9 (6:1) <sup>+</sup>	8.5 (4:1)	3.3 (1:3)	ND
	10 <sup>4</sup>	1.9 (4:1)	7.2 (3.5:1)	2.7 (1:4)	ND
MSV C3H2	0	ND	7.1 (3.5:1)	29.7 (3:1)	50.8 (2.5:1)
	10 <sup>4</sup>	ND	7.1 (3.5:1)	22.8 (2.2:1)	33.6 (1.6:1)
MSV C3H6	0	ND	8.0 (4:1)	32.0 (3.2:1)	48.8 (2.5:1)
	10 <sup>4</sup>	ND	5.8 (3:1)	22.6 (2.2:1)	22.2 (1.1:1)
MSV MEF2	0	ND	8.3 (4:1)	43.8 (4.4:1)	57.6 (2.8:1)
	10 <sup>4</sup>	ND	7.1 (3.5:1)	36.2 (3.6:1)	33.9 (1.7:1)

Effect of Interferon on DNA Synthesis at Different CellDensities

<sup>+</sup> Nos. in brackets indicate the ratio of cpm per cell.

Not Done : ND

per cell throughout ( $3$  to  $4 \times 10^3$  cpm per cell at 2000 cells/well, falling only to  $2.5$  to  $2.8 \times 10^3$  cpm per cell at 20000 cells per well). Interferon had a proportionally greater inhibitory effect at the higher cell density, the thymidine incorporated per cell being virtually unaltered at 2000 cells per well, but roughly halved at 20000 cells per well ( $1.1$  to  $1.7 \times 10^3$  cpm per cell). Nevertheless, DNA synthesis in the transformed cells at high density was still quite high, whereas the complete inhibition of growth seen in the growth curves of figure 7 would suggest that DNA synthesis should be reduced to zero. There are several possible explanations for this contradiction, the two most likely being (i) cells that are already crowded are much less sensitive to the addition of interferon than cells which are initially sparse at the time of interferon addition, or (ii) interferon takes more than 24 hours to cause maximal inhibition of DNA synthesis. These possibilities will be discussed in detail later.

#### D) Focus Formation

The ability of dispersed transformed cells to form discrete foci on top of a monolayer of normal cells is a growth property peculiar to the transformed phenotype, made possible by the lack of contact inhibition of movement and density dependent inhibition of growth in such cells. The effects of interferon on the ability of transformed non-



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producer cells to form foci was studied to assess interferon's efficiency in inhibiting growth under transformation-specific conditions. It was found that focus formation was inhibited by widely varying degrees, ranging from complete inhibition to only 50% reduction (Table 7). MSV C3H6 cells were not used in this experiment since they produce MSV/MLV and so form foci by spread of virus rather than by simple growth of cells, and since interferon inhibits MSV/MLV production (291) it would be difficult to dissociate this from an effect on cell growth. As with the growth curves, MSV MEF4 cells were the least sensitive to interferon, focus formation being reduced by only about 50%. However, CCl cells which were also quite insensitive to interferon's growth inhibitory activities, were clearly quite sensitive to its focus forming inhibitory activity, the numbers of foci formed being reduced by 96%.

This would

suggest that interferon's growth inhibition and inhibition of focus formation may occur by different mechanisms.

Experiments designed to distinguish between cell growth inhibition and a transformation-specific effect by interferon are described in section F.

#### E) Anchorage Independence

Transformed cells, having lost the requirement to attach to a solid substrate in order to grow, are able to grow quite freely when in suspension. Thus, growth of single

TABLE 7

Cell type	IFN (U/ml)	% Cloning in liquid medium	% Focus formation	Ratio of foci/clones efficiency	% Agar colonies formation	Ratio of agar cols/clones
NIH 3T3 4E	0	36 ± 3.7 <sup>1</sup>	-	-	-	-
	10 <sup>4</sup>	33 ± 5.8	-	-	-	-
C3H 10T <sup>1</sup>	0	61 ± 11.5	-	-	-	-
	10 <sup>4</sup>	25 ± 2.7	-	-	-	-
MSV C3H2	0	35 ± 9.0	32 ± 3.0	0.91	8.0 ± 1.2	0.23
	10 <sup>4</sup>	33 ± 10.4	0	0	3.3 ± 0.2	0.10
MSV C3H6	0	27 ± 11.0	-	-	21.2 ± 1.6	0.79
	10 <sup>4</sup>	18 ± 7.0	-	-	12.7 ± 1.3	0.70
MSV MEF2	0	59 ± 12.1	40 ± 3.0	0.68	14.0 ± 1.3	0.24
	10 <sup>4</sup>	49 ± 10.0	12 ± 1.2	0.24	4.0 ± 0.6	0.08
MSV MEF4	0	42 ± 6.7	46 ± 4.2	1.09	43.0 ± 3.8	1.02
	10 <sup>4</sup>	30 ± 5.2	22 ± 2.1	0.73	20.7 ± 3.4	0.69
CC1	0	74 ± 3.8	52 ± 1.6	0.70	37.1 ± 5.5	0.50
	10 <sup>4</sup>	50 ± 11.2	2 ± 2.5	0.04	13.5 ± 3.2	0.27
cA12	0	57 ± 12.2	26 ± 8.8	0.46	34.9 ± 5.5	0.61
	10 <sup>4</sup>	27 ± 10.7	11 ± 4.2	0.40	13.3 ± 6.5	0.49

Effect of Interferon on Cloning Efficiency in Liquid Medium, on Focus Formation and on Growth in Soft Agar of Two Normal and Six MSV-Transformed Cell Clones

<sup>1</sup> The data presented in this Table are the means of results from nine Petri dishes which received the same treatment in 3 separate experiments.

cells to form colonies suspended in agar is a property specific to the transformed state. The effects of interferon on this parameter were, therefore, studied to assess its ability to inhibit growth under these transformation-specific conditions.

The ability of transformed cells to grow in agar varied widely, ranging from only 8% efficiency for MSV C3H2 to 43% for MSVMEF4. Several of the transformed clones, e.g. MSV C3H2 and MSV MEF2, were unable to grow in agar at all at early passages post-transformation, but they were all able to grow in agar by passage 20 (292). The effects of interferon on efficiency of growth in agar were measured between the twentieth and thirtieth passages. It was found that interferon reduced the efficiency of growth by between 40% for MSV C3H6 and 71% for MSV/<sup>MEF2</sup>. Even with such a large inhibition, the efficiency of growth in an interferon-treated culture of CCl cells was still greater than growth in a control culture of MSV C3H2 cells, the efficiencies being 13.5% and 8% respectively (Table 7).

In addition those colonies that did grow in the presence of interferon were greatly reduced in size, compared to those in control cultures, and necrotic colonies were most frequently seen.

These data do not give an indication of the mechanism by which interferon inhibited focus formation and growth in agar. It is possible that the inhibition was due solely to

interferon's antiproliferative activity, but it is also possible that there may be a specific effect on the transformed phenotype. Experiments described in the next section clarify this possibility.

#### F) Cloning Efficiency in Liquid Medium

Growth of dispersed cells to form colonies on a solid surface in liquid medium is a property not specific to the transformed state and so inhibition of colony formation by interferon treatment under these conditions is likely to involve only the non-specific growth inhibitory activity. Experiments were conducted to measure the efficiency of cloning on plastic, and these data were then used to identify a transformation-specific effect of interferon on focus formation and growth in agar by transformed cells.

Table 7 shows that interferon had variable effects on the cloning efficiency of normal and transformed cells, ranging from a 59% reduction for C3H10T $\frac{1}{2}$  cells to little or no change for NIH 3T3 and MSV C3H2 cells. In most cases the sizes of the colonies which did form were also somewhat reduced by interferon.

When the efficiency of cloning of transformed cells on plastic was compared to their growth in agar and focus formation, it was found that interferon did have a greater inhibitory effect on these two transformation-specific parameters. This was measured by calculating the ratios for efficiency

of focus formation or growth in agar to the efficiency of cloning on plastic.

For focus formation interferon treatment reduced the ratio by a variable degree, ranging from 33% for MSV MEF4 cells, to a reduction to zero for MSV C3H2 cells due to the total inhibition of focus formation in these cells. With growth in agar the reductions in the ratio were a little less dramatic, ranging from 11% for MSV C3H6 cells to 67% for MSVMEF2 cells.

These data indicate that the two transformation-specific properties of anchorage independence and focus formation were inhibited by interferon by at least two mechanisms - a non-specific growth inhibition, and an additional activity which was highly variable but accounted for a considerable proportion of the inhibitory activity against focus formation and growth in agar. Since interferon was much more active at inhibiting growth of transformed cells under conditions selective for the state than it was under non-selective conditions, it would seem very likely that the additional activity in transformation-selective conditions was due to an effect aimed specifically against the transformed phenotype.

#### G) Effects of Serum Concentration on Interferon Activity

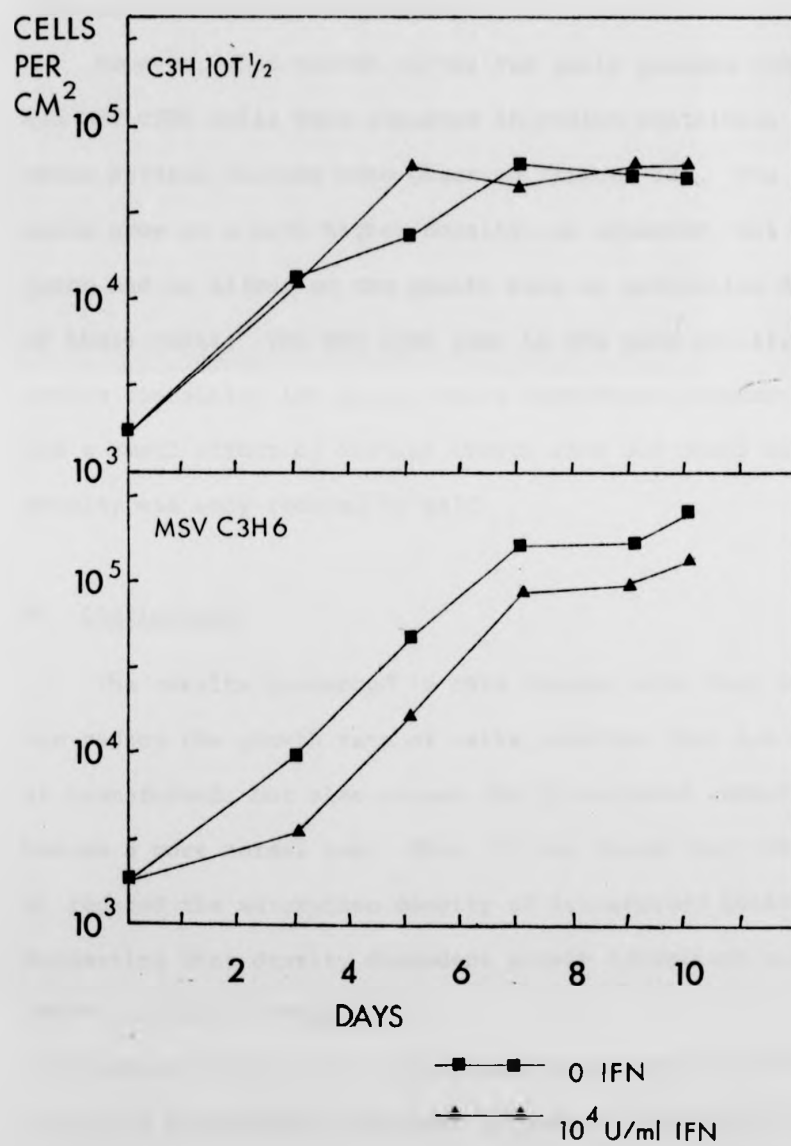
Kading et al. (138) reported that lowering the serum concentration enhanced interferon's inhibition of cloning in liquid media. However, using  $10^4$  U/ml and  $3 \times 10^4$  U/ml of

TABLE 8

Serum Content of Medium (%)	IFN (U/ml)	Cloning Efficiencies in Liquid Medium (%)		
		CC1	ca12	DA4
20	0	39	51	27
	$10^4$	48	39	33
	$3 \times 10^4$	42	33	20
10	0	ND	54	22
	$10^4$	ND	50	19
	$3 \times 10^4$	ND	42	20
5	0	28	34	20
	$10^4$	33	37	18
	$3 \times 10^4$	24	26	17

Effects of Serum Content of Medium on Interferon's Inhibition  
of Cloning on Plastic

Not Done: ND

FIGURE 10

Effect of Interferon on Growth and Saturation Densities of  
Cells Grown in Medium Containing a Very High Serum Content.



interferon no change in interferon's effect on cloning could be observed on these cells when the serum concentration was lowered from 20% to 5% (Table 8).

However, when growth curves for early passage C3H10T $\frac{1}{2}$  and MSV C3H6 cells were repeated in medium containing 50% serum several changes were observed (figure 10). The C3H10T $\frac{1}{2}$  cells grew to a much higher density, as expected, but interferon had no effect on the growth rate or saturation density of these cells. The MSV C3H6 grew to the same density as in medium containing 10% serum, while interferon treatment only had a small effect on initial growth rate and final saturation density was only reduced by half.

#### H) Conclusions

The results presented in this chapter show that interferon can reduce the growth rate of cells, whether they are normal or transformed, but also causes the transformed phenotype to become a more normal one. Thus, it was found that interferon;

- a) reduced the saturation density of transformed cells, suggesting that density dependent growth inhibition was at least partially restored;
- b) inhibited ability of single transformed cells to form foci on top of a confluent monolayer of normal cells, which even after growth inhibition is taken into account, suggests at least partial restoration of contact inhibition of movement, and density dependent inhibition of growth;

c) reduced the ability of transformed cells to grow suspended in agar, which again even when growth inhibition has been accounted for, suggests that partial restoration of anchorage dependence occurred.

Serum factors may influence these actions of interferon, overcoming its growth inhibitory activity at very high concentrations but having little effect on interferon's inhibition of colony formation at lower concentrations.

### CHAPTER THREE

#### INTERACTIONS BETWEEN BUTYRIC ACID AND INTERFERON IN GROWTH INHIBITION

It has been known for some time that sodium butyrate could inhibit the growth of many cell types and could also cause partial reversal of the transformed phenotype (8, 83, 85, 171). Transformed cells become flatter, grow in more orderly arrays and possess a near-normal microfilament system (8, 171, 243), but do not show density-dependent inhibition of growth (8, 83).

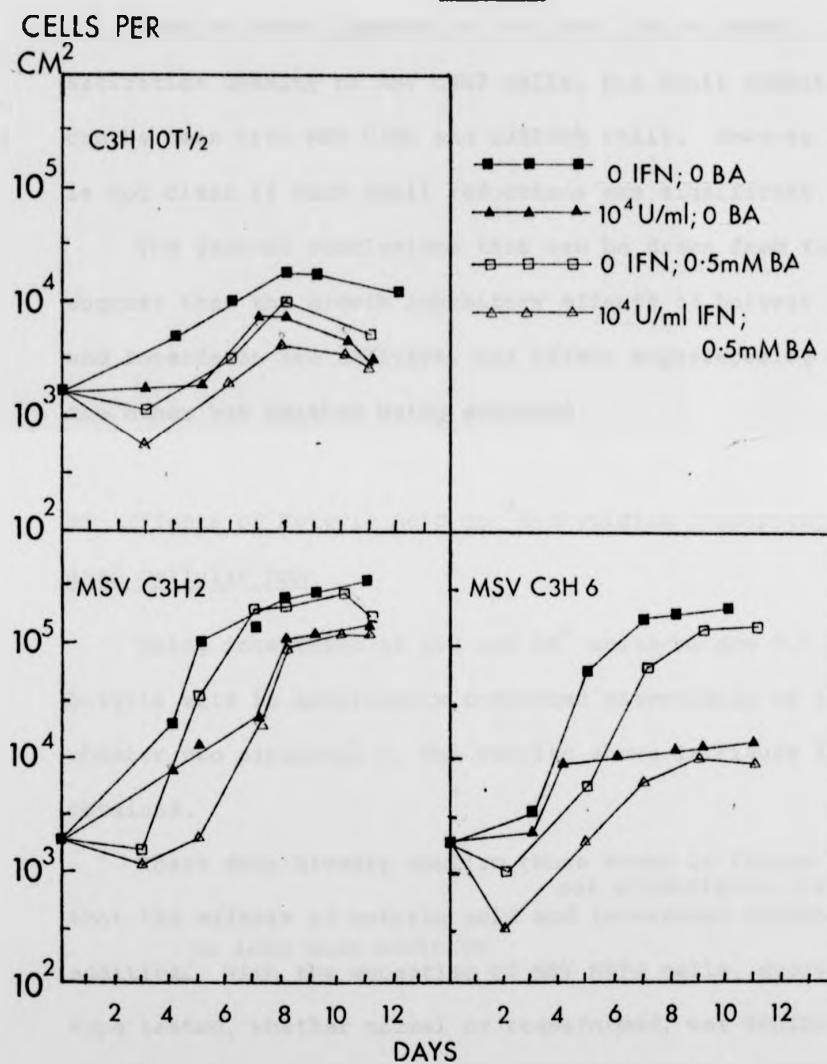
Bourgeade and Chany (35) recently reported that sodium butyrate could enhance interferon's antiviral and growth inhibitory activities on MSV-transformed cells, but not on normal embryonic fibroblasts.

This chapter describes studies on the effects of butyric acid on interferon's growth inhibitory activity, while chapter 4 looks at the effects on cell morphology.

#### A) Effects of Butyric Acid on Growth Curves

Figure 11 shows the growth curves obtained as a result of treatment with interferon and butyric acid on C3H10T $\frac{1}{2}$ , MSV C3H2 and MSV C3H6 cells. The greatest effect of 0.5 mM butyric acid both on MSV-transformed and normal cells was to greatly delay initiation of growth, but once the logarithmic phase started, growth appeared to have been almost as rapid as in controls. This inhibition was even more severe when butyric acid and

FIGURE 11



Effects of Butyric Acid, in the Presence or Absence of Interferon, on the Growth of Cells.

interferon were added together.

Butyric acid appeared to have had little effect on the saturation density of MSV C3H2 cells, but small reductions can be seen with MSV C3H6 and C3H10T $\frac{1}{2}$  cells. However, it is not clear if such small reductions are significant.

The general conclusions that can be drawn from fig 11 suggest that the growth inhibitory effects of butyric acid and interferon are additive, one effect superimposing upon the other but neither being enhanced.

#### B) Effects of Butyric Acid on $^3\text{H}$ -thymidine Incorporation into Cellular DNA







Using interferon at 100 and  $10^4$  units/ml and 0.5 mM butyric acid in experiments conducted essentially as in chapter two paragraph C, the results shown in Figure 12 were obtained.

These data broadly confirm those shown in Figure 11; not synergistic, but either that the effects of butyric acid and interferon together are~~x~~ or less than additive~~x~~ additive~~x~~. With the exception of MSV MEF4 cells, every cell type tested, whether normal or transformed, was inhibited by 0.5 mM butyric acid and 100 units/ml interferon at most cell densities. The two agents usually inhibited  $^3\text{H}$ -thymidine incorporation by as much as  $10^4$  units/ml interferon alone. MSV MEF4 cells appeared to be almost completely resistant to butyric acid, but did show some sensitivity to interferon (though less than any of the other cell types tested).

FIGURE 12 Effect of Interferon and Butyric Acid on DNA

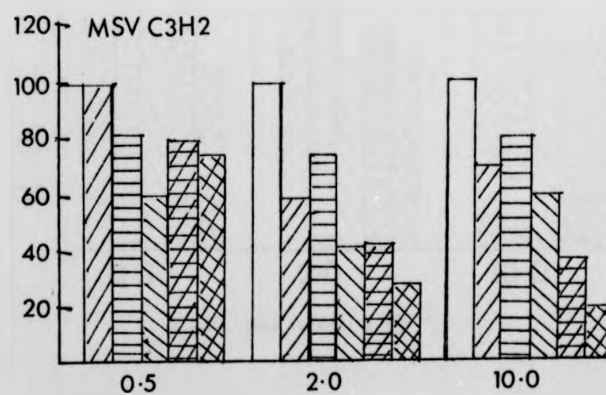
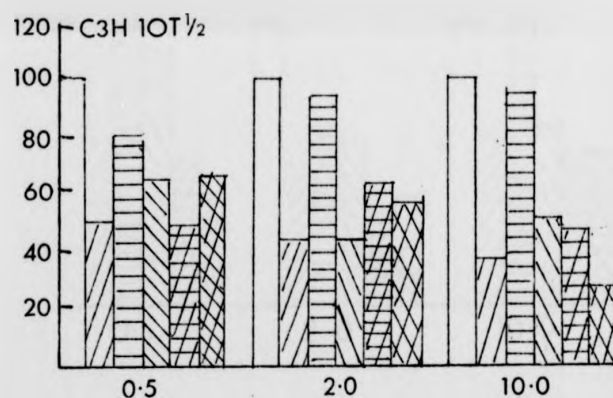
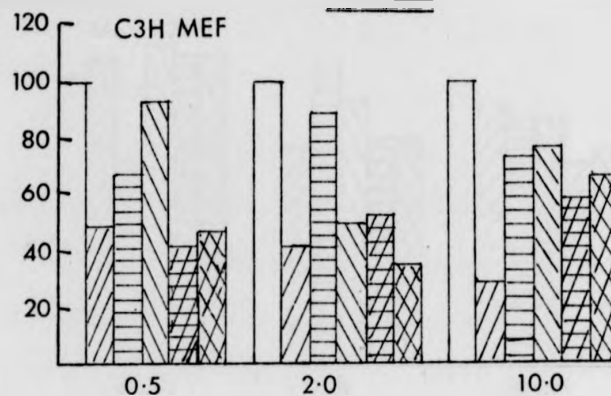
Synthesis of Cells at Different Densities

KEY

	0 IFN; 0 BA (control)
	0 IFN; 0.5mM BA
	$10^2$ U/ml IFN; 0 BA
	$10^4$ U/ml IFN; 0 BA
	$10^2$ U/ml IFN; 0.5mM BA
	$10^4$ U/ml IFN; 0.5mM BA

Interferon and butyric acid (BA) treatments lasted for 24 hours prior to labelling with  $^3\text{H}$ -thymidine.

The results are expressed as percentage of radioactivity incorporated into each sample compared to that incorporated into the control sample.

as % of  
controlsCells per well ( $\times 10^{-3}$ )

Effect of Interferon and Butyric Acid on DNA Synthesis of Cells  
at Different Densities

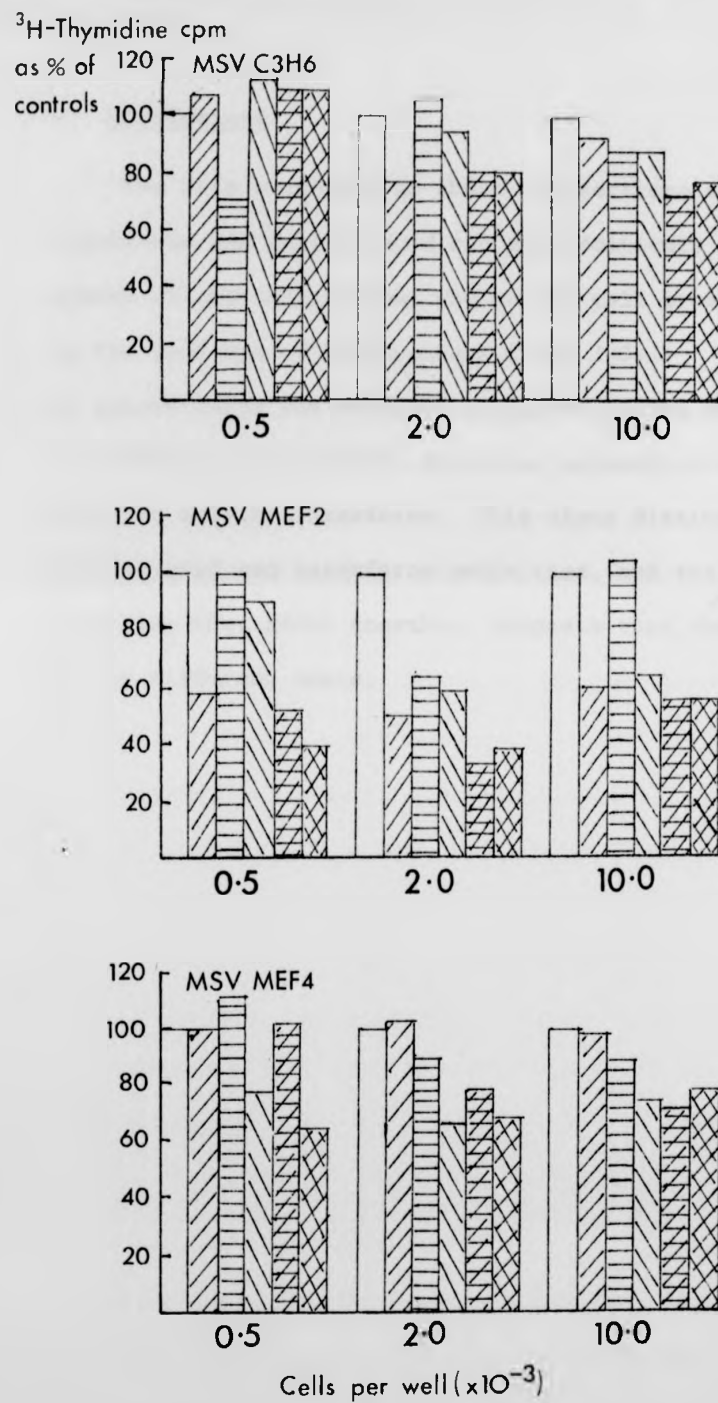


FIGURE 12 cont/d.



C) Conclusions

The data presented in these two sections show that interferon and butyric acid can act additively to inhibit growth of both transformed and normal cell types (in contrast to the findings of Boumeade and Chany (35) ). Thus growth of sparse cells was severely inhibited by the additive effects of interferon and butyric acid, but saturation density was affected only by interferon. This clear distinction between butyric acid and interferon activities, and their lack of synergism when added together, suggests that the two agents act by different means.

## CHAPTER FOUR

### EFFECTS ON CELL SURFACES AND MORPHOLOGY

Cell-to-cell and cell-to-substrate interactions are very important in the control of cell behaviour, organisation and growth both in vivo and in vitro. Transformed and tumour cells show many changes at their surfaces which may be linked to the loss of some characteristics of normal cells such as a well-spread flattened morphology, strong adhesion to the substrate and density dependent inhibition of growth.

A series of experiments were conducted to assess whether interferon's transformation-specific activity could be attributed to some effects on the cells' surfaces, resulting in a more normal behaviour. Agglutinability by concanavalin A, and adhesion to glass surfaces were briefly examined, but the majority of the studies concentrated on observing the overall cellular morphology, intracellular microfilament system and the cell surface fibronectin distribution. Butyric acid's combined effect with interferon on these parameters was also studied.

#### A) Agglutinability by Concanavalin A

A number of experiments were conducted in which the agglutinability of CCl cells grown in the presence or absence of  $10^4$  U/ml interferon was compared with that of NIH 3T3 cells. Table 9 shows the results of the first experiment in which the CCl cells grown in the presence of interferon were clearly

TABLE 9

CELL LINE	IFN (U/ml)	Con A ( $\mu$ g/ml)	% Cells not Agglutinated after Time (mins)		
			0 mins	15 mins	30 mins
NIH 3T3 4E	0	0	100	100	94
		100	100	38	34
CCl	0	0	100	100	100
		100	100	22	7
	10 <sup>4</sup>	0	100	100	100
		100	100	66	54

Agglutinability of CCl and NIH 3T3 4E Cells by Con A after Growth  
in the presence or Absence of Interferon

much less agglutinable than those grown without interferon, or even than NIH 3T3 cells. Several subsequent experiments gave much less clear-cut results, only a two-fold reduction in agglutinability being seen (results not shown). These variable results were attributed at least in part to the technical problems involved in this experiment (such as preventing cells adhering to the walls of the vessel), and so these studies were not continued. Nevertheless, the data presented in Table 9, and general observations of the behaviour of trypsinised cells strongly suggest that interferon-treated transformed cells are less agglutinable than those grown in the absence of interferon.

#### B) Morphology

The majority of transformed cell types show a different morphology from their normal parents, being small and rounded, and the cultures do not possess the orderly whorls and parallel arrays of cells typical of normal fibroblast cultures, but grow in a totally disorderly manner. There have been numerous reports that agents which reverse the transformed phenotype, such as cyclic AMP and butyric acid, cause the cells to be flatter and adopt more orderly patterns (8, 83, 205, 243). Only a few studies have reported that interferon has such an effect on morphology (78, 89, 201). A series of experiments was therefore conducted to assess whether interferon could affect the morphology of these cells, after

treatment for varying periods up to one week.

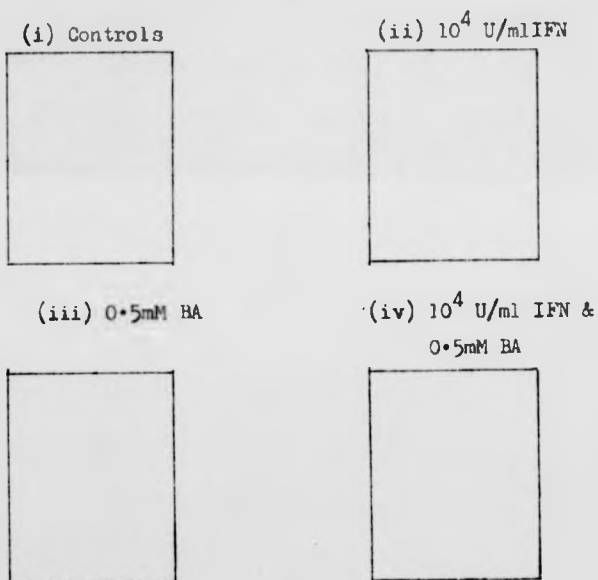
Figure 13 illustrates that in the presence of  $10^4$  units/ml of interferon cells were enlarged. In this figure the morphologies of six cell types with or without interferon and/or butyric acid are presented. These cell types are NIH MEF, C3H MEF, C3H10T $\frac{1}{2}$ , MSV C3H2, MSV C3H6, MSV MEF2 and MSV MEF4. For each cell type there are four photographs: i) control, ii) with  $10^4$  units/ml interferon, iii) with 0.5 mM butyric acid and iv) with 0.5 mM butyric acid and  $10^4$  units/ml of interferon.

As with the growth curves, the NIH MEF cells appeared to be resistant to the interferon, remaining at about 200-400  $\mu$ m long (Fig. 13b (i) and (ii)), while the C3H MEF cells increased in size from about 100-200  $\mu$ m long to 250-400  $\mu$ m in length (Fig. 13a (i) and (ii)). The C3H10T $\frac{1}{2}$  cells, in the absence of interferon were approximately the same size as untreated C3H MEF cells, but in its presence they appeared to respond less dramatically than did the primary embryonic fibroblasts, increasing in length to only about 300  $\mu$ m (fig. 13c (i) and (ii)). Cells of the different transformed clones shown in figure 13d-g clearly became flattened and more orderly, except for MSV MEF4 cells which appeared to be quite insensitive to interferon (fig. 13g (i) and (ii)). This insensitivity of MSV MEF4 cells correlates with their resistance to interferon's growth inhibition. MSV C3H2 and 6 and MSV MEF2 cells all increased their sizes from 50-100  $\mu$ m

# LEGEND TO FIGURE 13

The effects of interferon and butyric acid on cell and culture morphology.

Cells grown on plastic petri dishes with or without  $10^4$  U/ml interferon and 0.5mM butyric acid; fixed with 3.5% formaldehyde in PBS and stained with crystal violet. Arrangement of plates is as follows:-



Plates: a) C3H MEF; b) NIH MEF; c) C3H 10T $\frac{1}{2}$ ; d) MSV C3H2; e) MSV C3H6; f) MSV MEF2; g) MSV MEF4.

Scale:

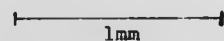


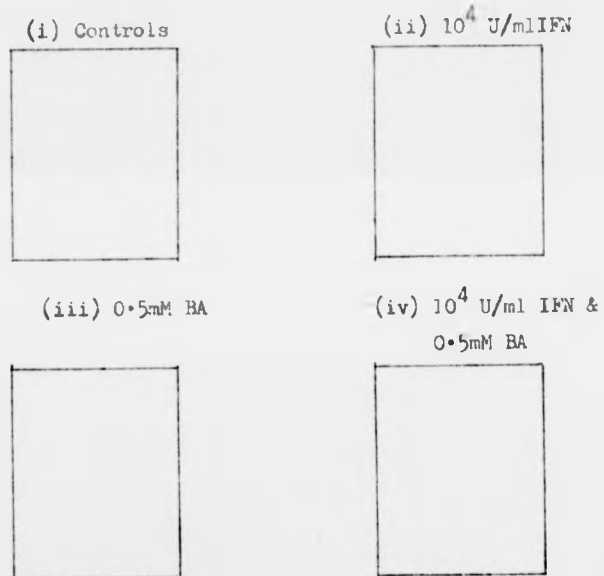
FIGURE 13a



# LEGEND TO FIGURE 13

The effects of interferon and butyric acid on cell and culture morphology.

Cells grown on plastic petri dishes with or without  $10^4$  U/ml interferon and 0.5mM butyric acid; fixed with 3.5% formaldehyde in PBS and stained with crystal violet. Arrangement of plates is as follows:-



Plates: a) C3H MEF; b) NIH MEF; c) C3H 10T $\frac{1}{2}$ ; d) MSV C3H2; e) MSV C3H6; f) MSV MEF2; g) MSV MEF4.

Scale:

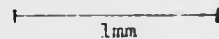


FIGURE 13a

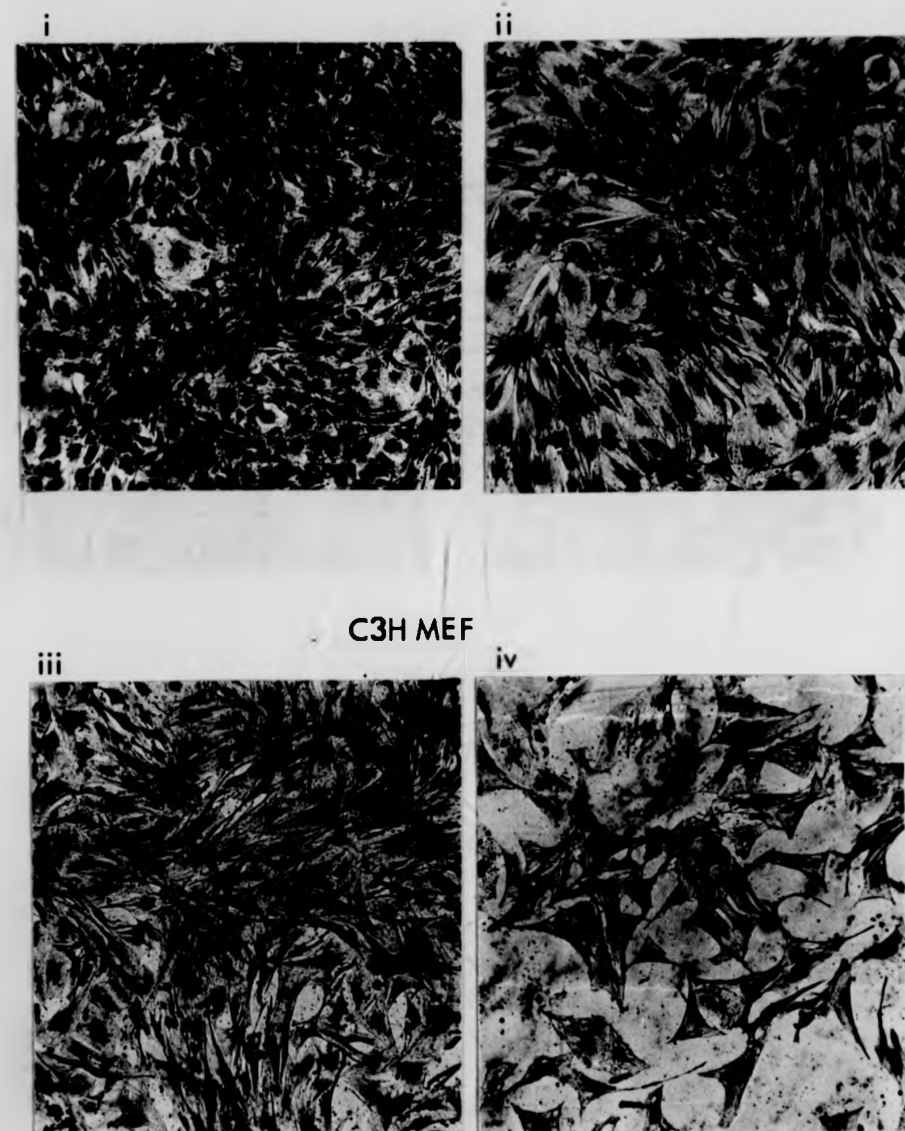
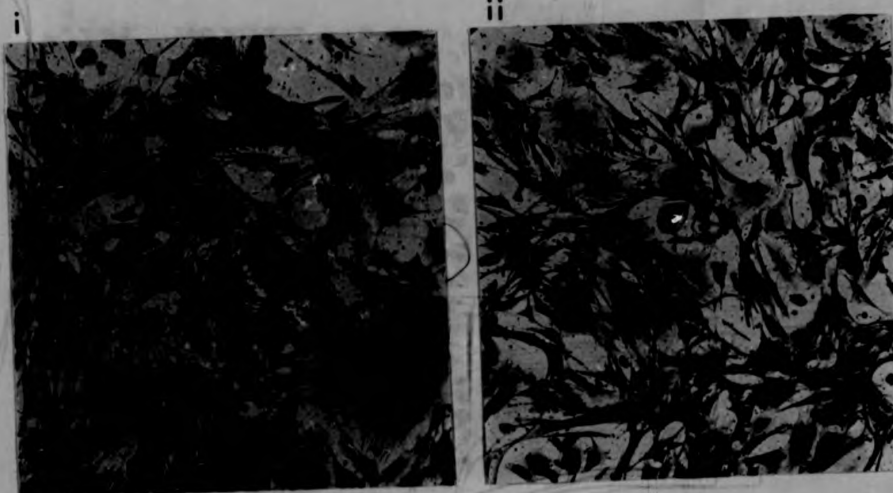




FIGURE 13b



NIH MEF





FIGURE 13b



NIH MEF

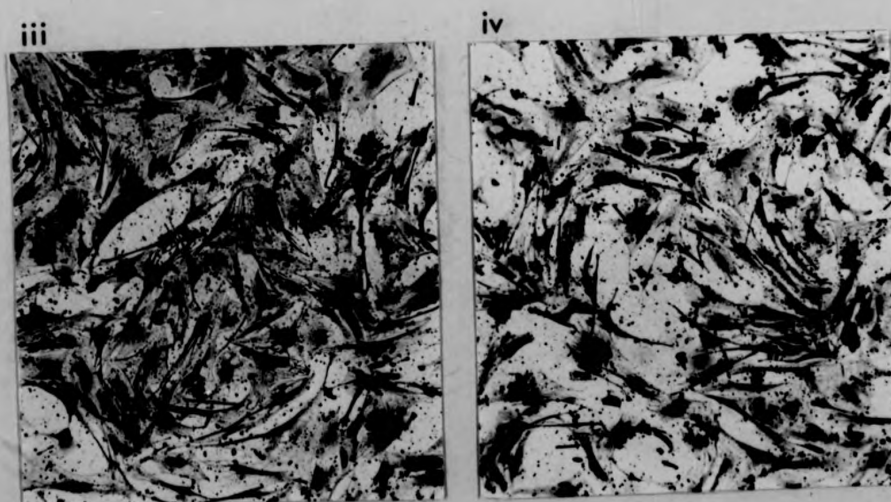


FIGURE 13c



FIGURE 13c

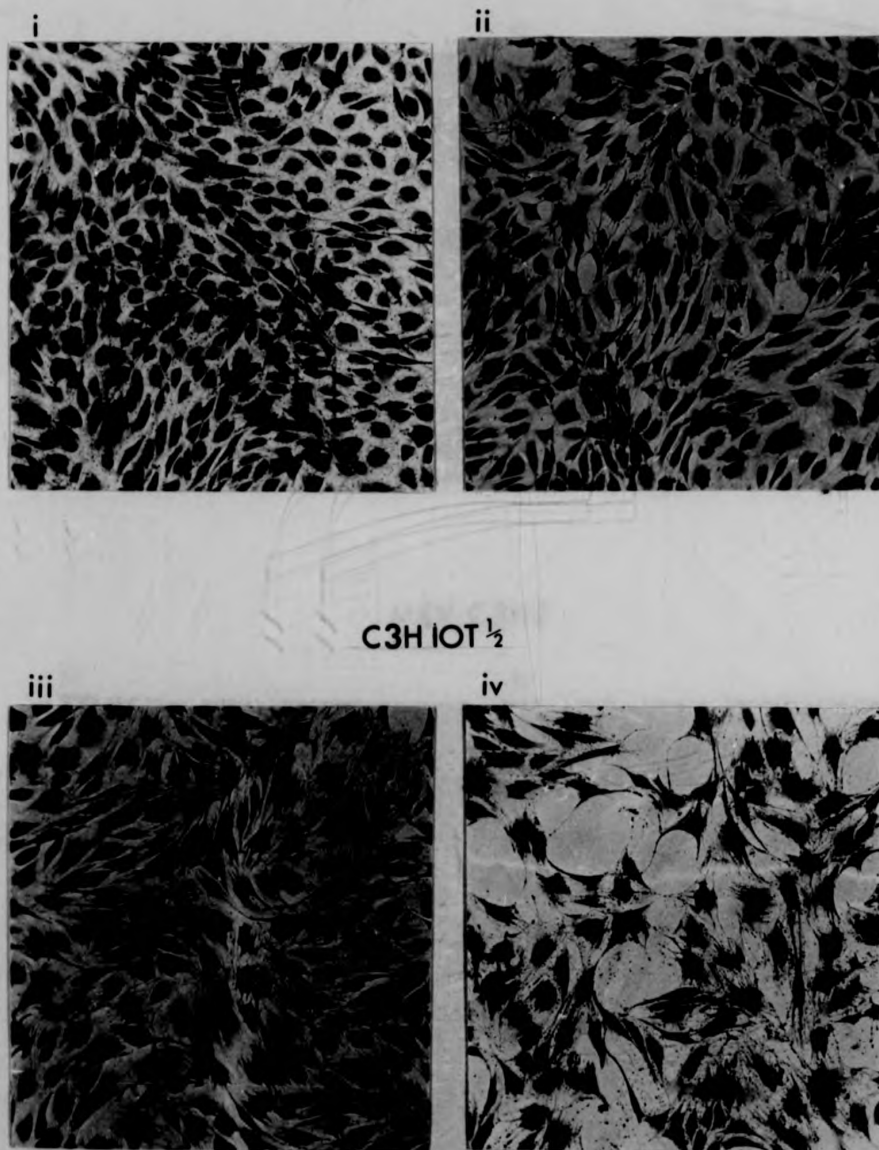
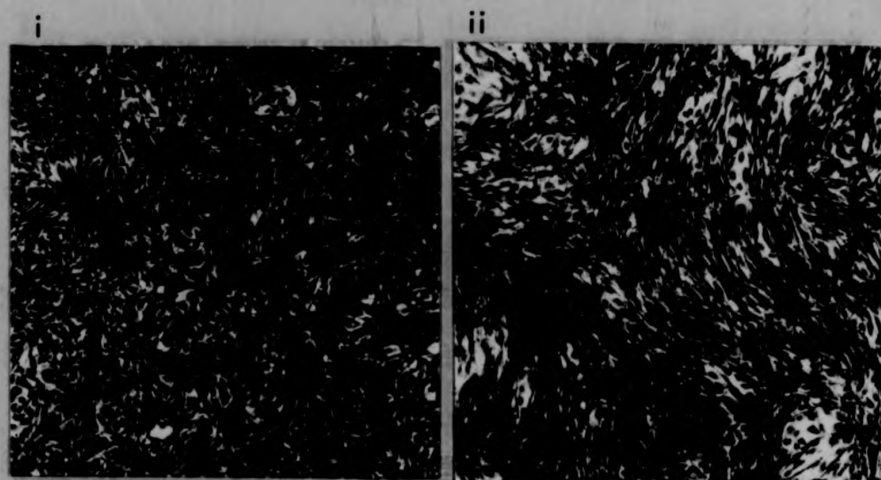


FIGURE 13d



MSV C3H2

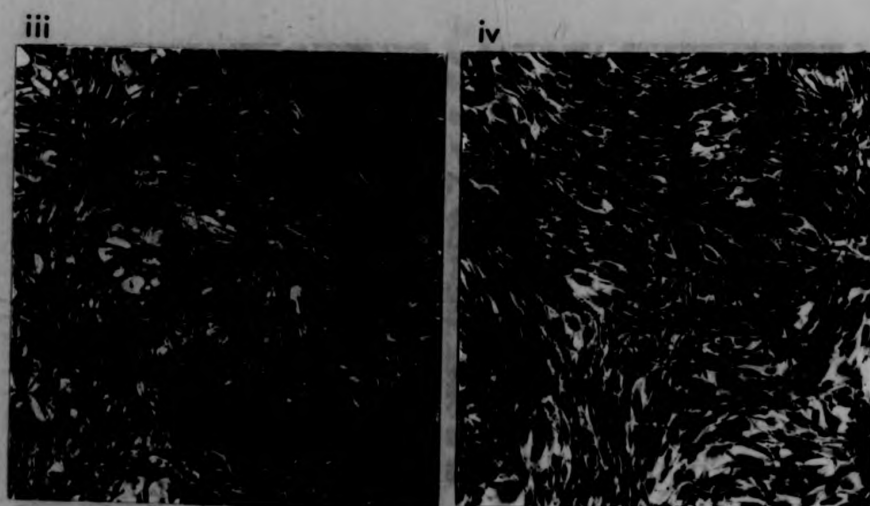
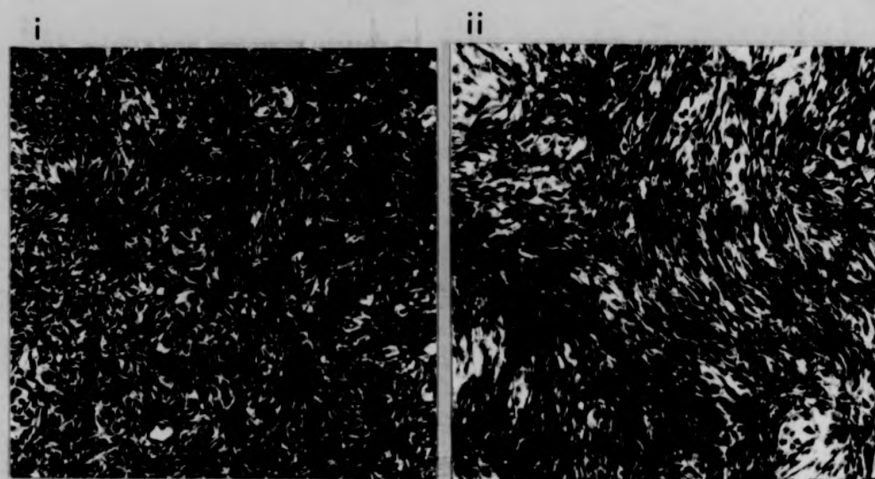




FIGURE 13d



MSV C3H2

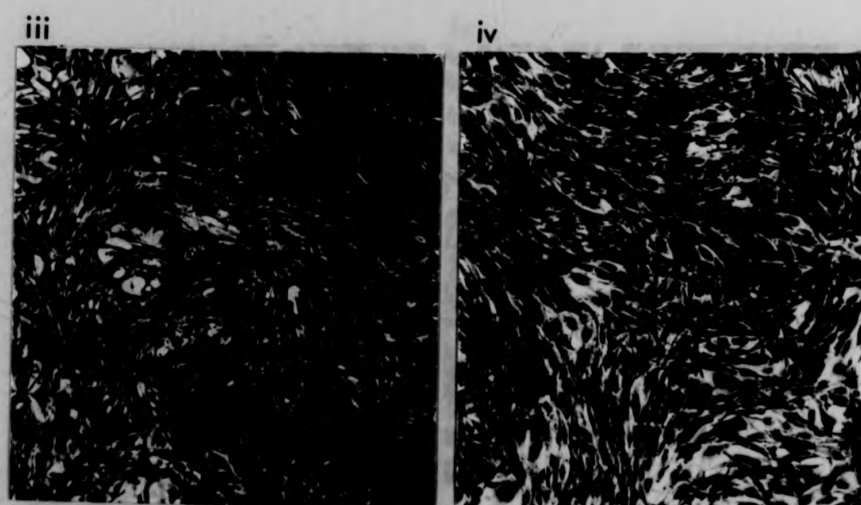


FIGURE 13e

i



ii



MSV C3H6

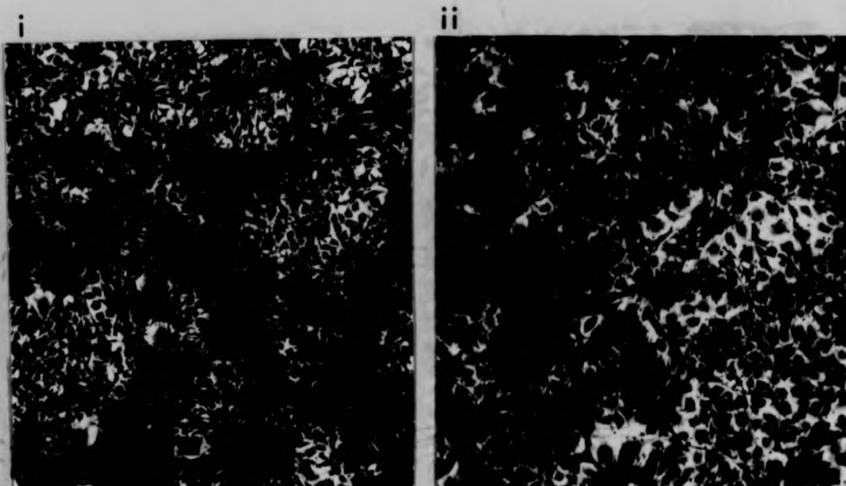
iii



iv



FIGURE 13e



MSV C3H6

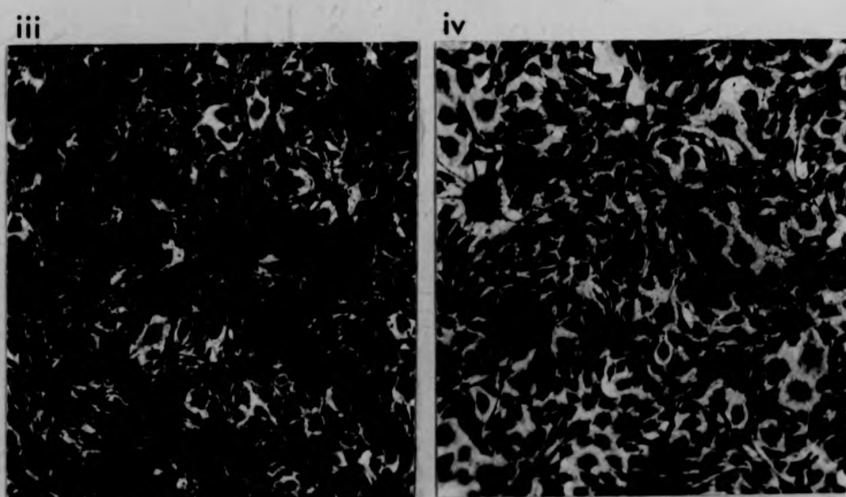
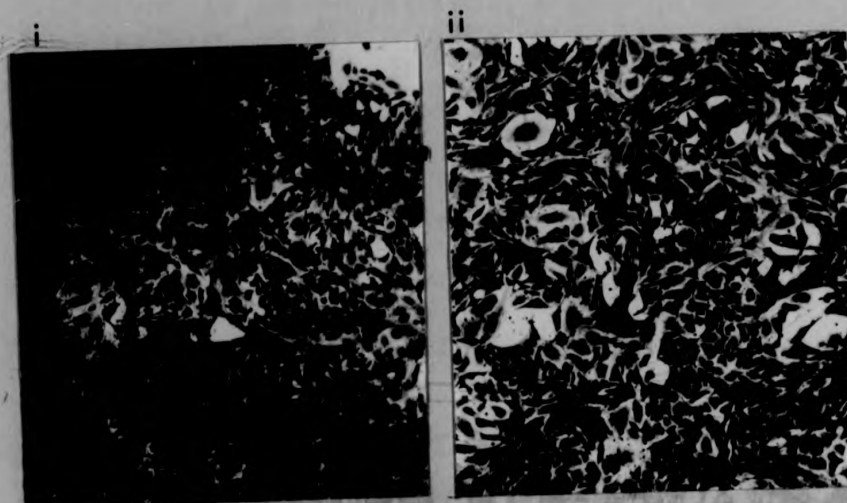


FIGURE 13f



MSV MEF 2

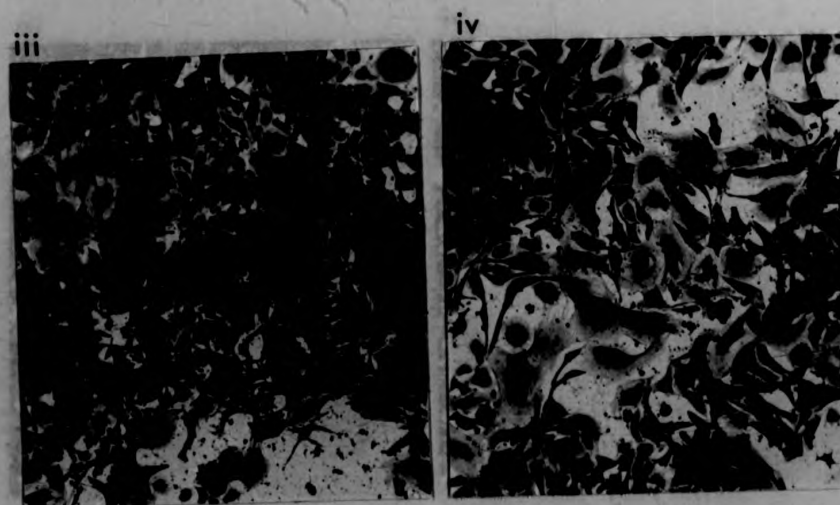
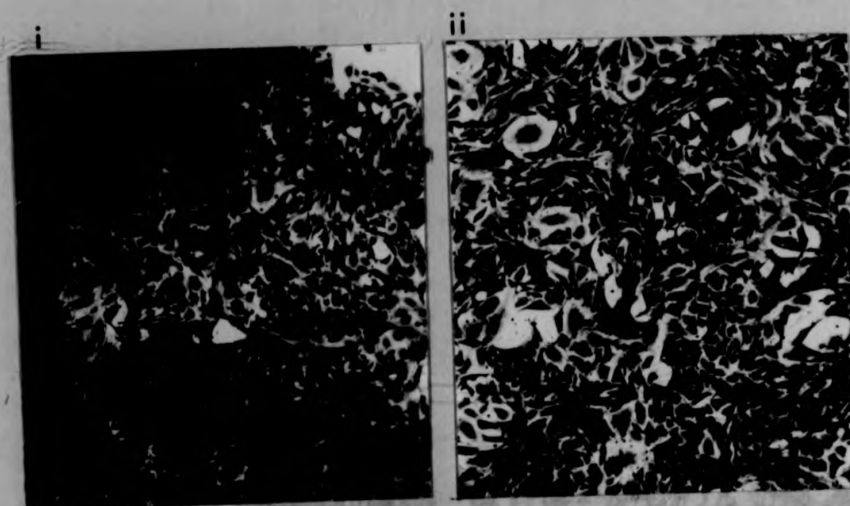




FIGURE 13f



MSV MEF 2

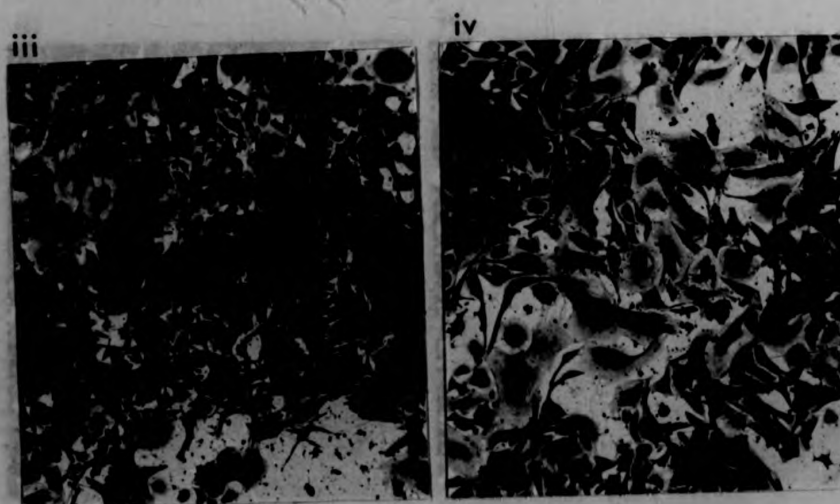
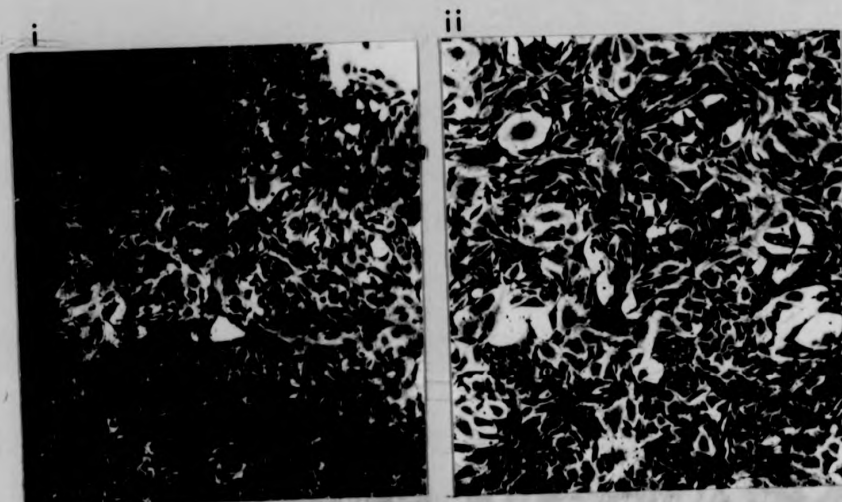


FIGURE 13f



MSV MEF 2

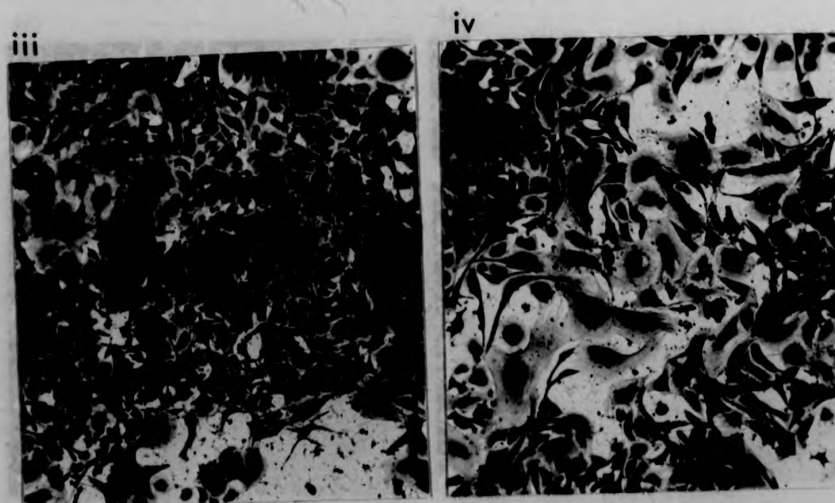
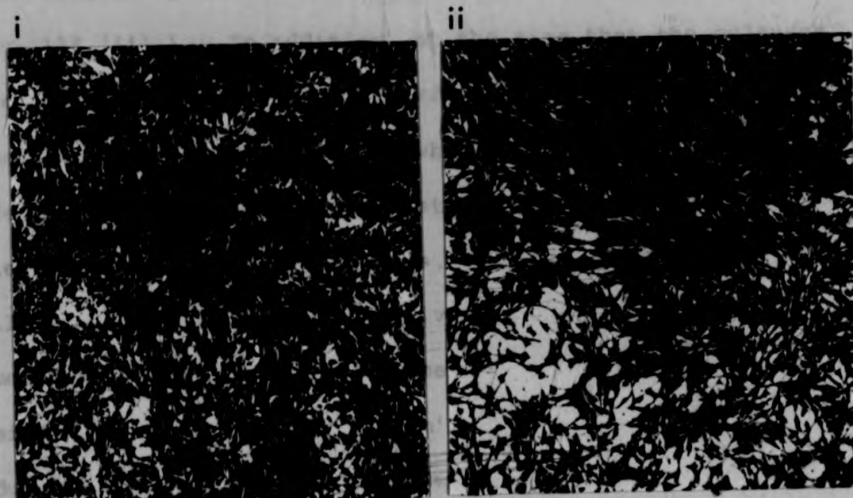
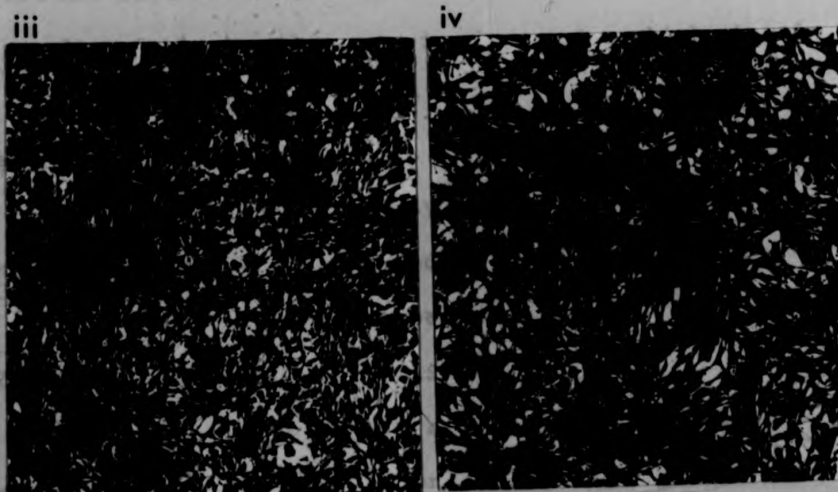


FIGURE 13g

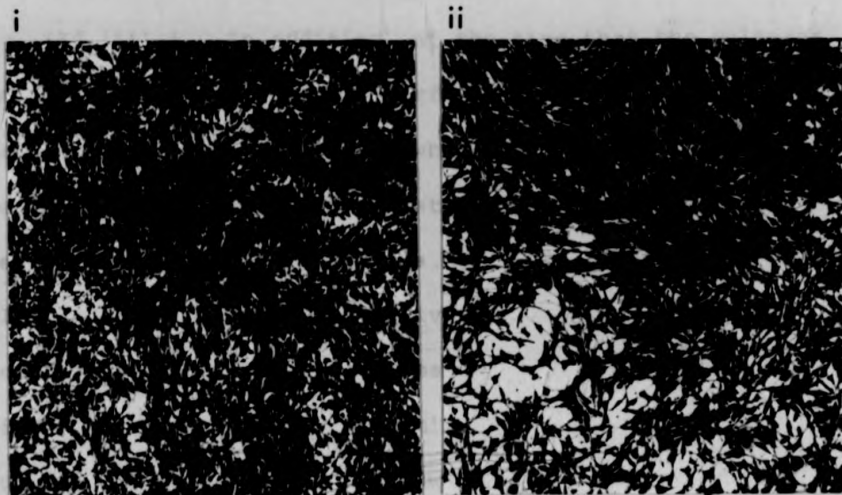


Increased spreading of cells would be seen with as little as 10 units/ml of **MSV MEF 4** but not MSV CH2 cells. Results not shown, which parallels

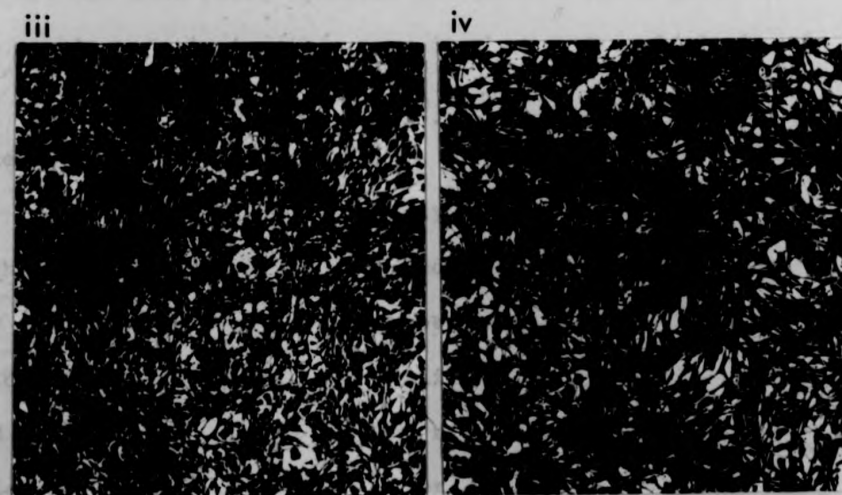


together indicated further the role of MSV MEF 4 in the spreading of cells. With some MSV MEF 4 cells reaching up to 400  $\mu$ m long, with the

FIGURE 13g



MSV MEF4



in length to yield a heterogeneous mixture of cells ranging from 80  $\mu\text{m}$  to 200  $\mu\text{m}$  long, the largest cells only being found frequently in the interferon-treated MSV MEF2 cultures (fig. 13f (ii) ). In addition, at the time that the cultures were fixed and stained, the interferon-treated cultures were close to their maximum density, while the untreated controls were still growing rapidly, and still sparse enough to be able to identify individual cells. NIH 3T3 4E, CCl and cAl2 cells, like MSV MEF4, were relatively insensitive, though some flattening could be seen (results not shown), paralleling their insensitivity to interferon's saturation density reducing effect seen in chapter two.

Increased spreading of cells could be seen with as little as 10 units/ml of interferon on MSV C3H6 and MSV MEF2 cells, but not MSV C3H2 cells (results not shown), which parallels the results of the growth curves in chapter two which showed MSV C3H6 and MSV MEF2 cells to be the most sensitive to interferon of the clones studied.

Butyric acid (0.5 mM) clearly had a combined action with interferon on the transformed cells (fig. 13), except MSV MEF4 cells which once again were resistant to both butyric acid and interferon (fig. 13g (iii) and (iv) ). With MSV C3H2 and 6 and MSV MEF2 cells butyric acid treatment alone had a roughly similar effect to interferon alone, but the two together increased further the cells' size to 150-200  $\mu\text{m}$  long with some MSV MEF2 cells reaching up to 400  $\mu\text{m}$  long, with an



appearance very similar to untreated NIH MEF cells (fig. 13d-f). Furthermore, the cultures became very much more orderly, with the parallel arrays and whorls, typical of normal cultures, becoming prominent.

The combined effects of interferon and butyric acid were evident at interferon doses as low as 10 units/ml with MSV C3H6 and MSV MEF2, and the degree of cell flattening when in the presence of 100 units/ml interferon plus 0.5 mM butyric acid was quite similar to that observed in cultures treated with  $10^4$  units/ml interferon alone (results not shown).

With the normal cells (fig. 13a-c) there was less evidence of combined effects. There appeared to be very little effect on the morphology of NIH MEF cells by butyric acid alone or in conjunction with interferon. C3H MEF cells, on the other hand, did become enlarged by butyric acid alone, to roughly the same size as when treated with  $10^4$  units/ml interferon alone, but butyric and interferon together appeared to have little additional effect. Similar observations were made with C3H10T $\frac{1}{2}$  cells, though possibly interferon and butyric acid together did show a small combined effect (fig. 13c).

The main result of butyric acid treatment of these normal cells seems to have been a great inhibition of growth, which was more severe than in cultures of transformed cells.

#### C) Microfilament System

Reversion of rounded transformed morphology to a more

normal flattened state may be accompanied by at least a partial re-establishment of the microfilament system of the cytoskeleton. This possibility was studied by staining some cell types, grown with or without interferon, with FITC-DNase I. DNase I binds specifically to actin, in addition to DNA, and FITC or rhodamine-conjugated DNase has been reported to be an effective agent with which to stain microfilaments for visualisation under an ultraviolet fluorescent microscope (260, 261).

Figure 14 shows the results of experiments in which cells were grown on glass coverslips with or without  $10^4$  units/ml of interferon for three days before being fixed with 3.5% formaldehyde in PBS and stained with FITC-DNase I.

As expected, NIH MEF cells showed little change (fig. 14a and b). C3H10T $\frac{1}{2}$  cells showed a microfilament system poorly, but interferon treatment did appear to stimulate the establishment of a more organised system, though the observations were not unequivocal (fig. 14c and d). MSV C3H2 and MSV MEF2 cells, in the absence of interferon, generally showed few signs of microfilaments, but in its presence these became quite visible. Figure 14e shows MSV C3H2 cells without interferon, and in this picture microfilaments could only faintly be seen in one cell, while in the interferon-treated culture (fig. 14f) all cells showed microfilaments.

With MSV MEF2 cells the effect was less clear-cut. There were obviously no microfilaments in the absence of

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With MSV MEF2 cells the effect was less clear-cut. There were obviously no microfilaments in the absence of



LEGEND TO FIGURE 14

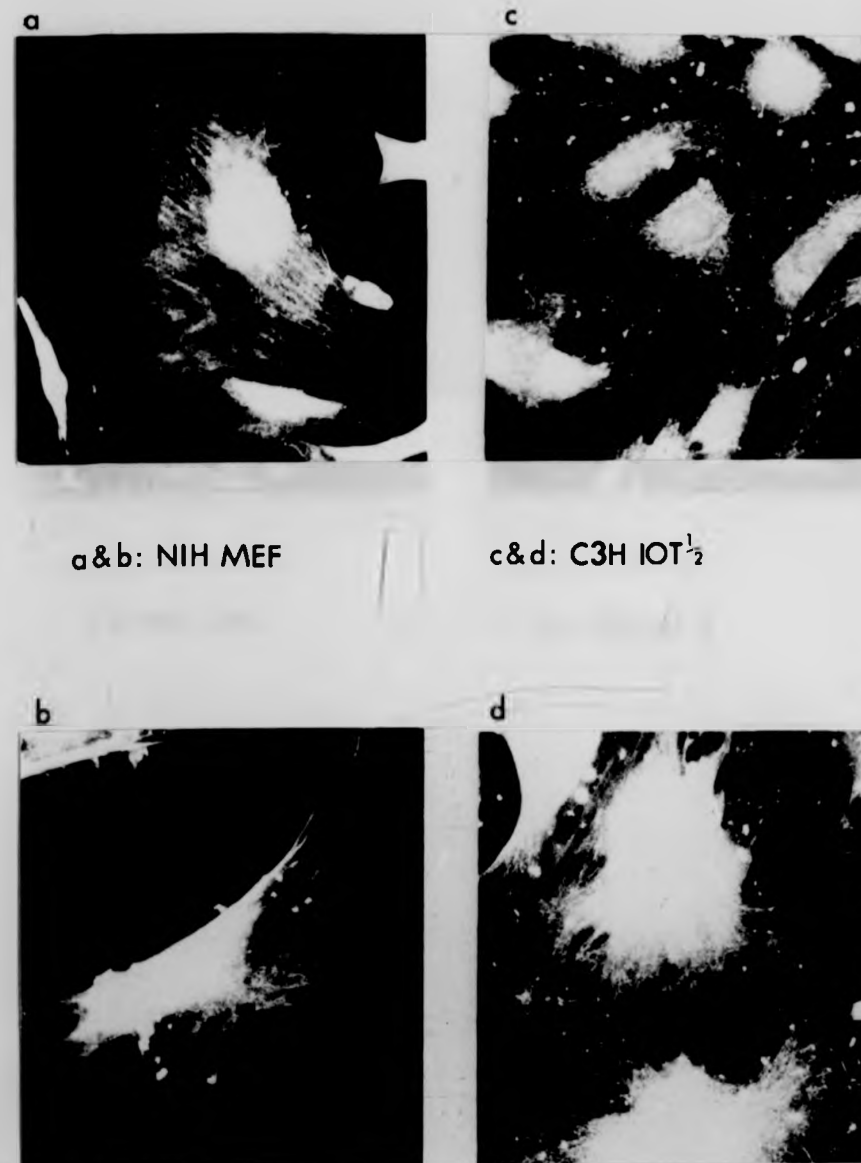
The effects of interferon on the microfilament system of normal and transformed cells. Cells were grown on glass coverslips with or without interferon, fixed with 3.5% formaldehyde in PBS, treated with cold methanol ( $-20^{\circ}\text{C}$ ) and stained with FITC-DNase I.

Plates;

a, c, e, g, i : No interferon (controls).

b, d, f, h, j :  $10^4$  U/ml interferon.

FIGURE 14



a&amp;b: NIH MEF

c&d: C3H 10T $\frac{1}{2}$

LEGEND TO FIGURE 14

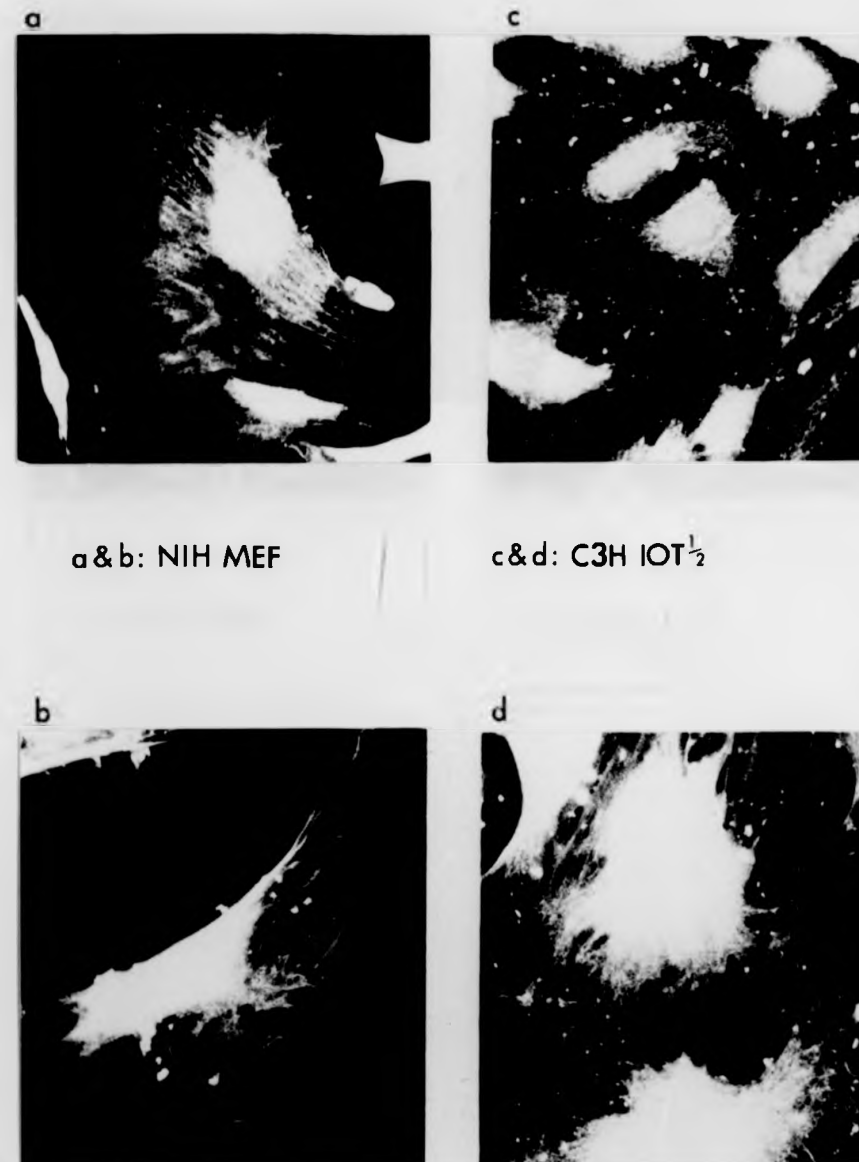
The effects of interferon on the microfilament system of normal and transformed cells. Cells were grown on glass coverslips with or without interferon, fixed with 3.5% formaldehyde in PBS, treated with cold methanol ( $-20^{\circ}\text{C}$ ) and stained with FITC-DNase I.

Plates;

a, c, e, g, i : No interferon (controls).

b, d, f, h, j :  $10^4$  U/ml interferon.

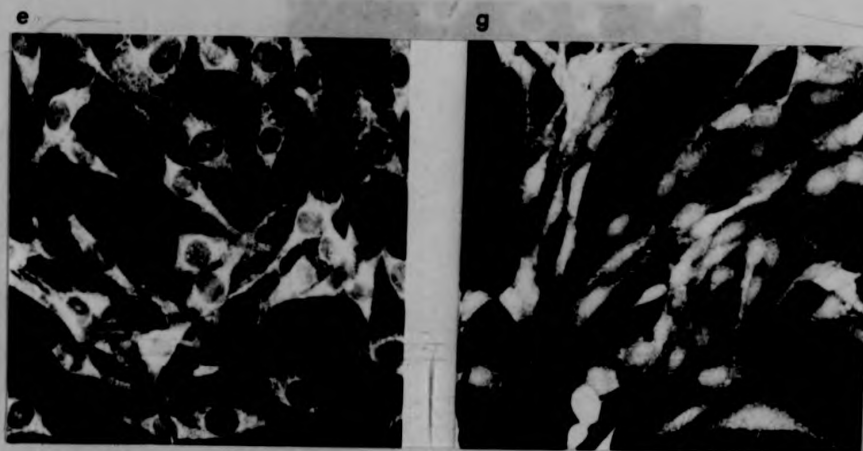
FIGURE 14



a&b: NIH MEF

c&d: C3H 10T $1/2$

FIGURE 14



e &amp; f MSV C3H2

g &amp; h MSVMEF2

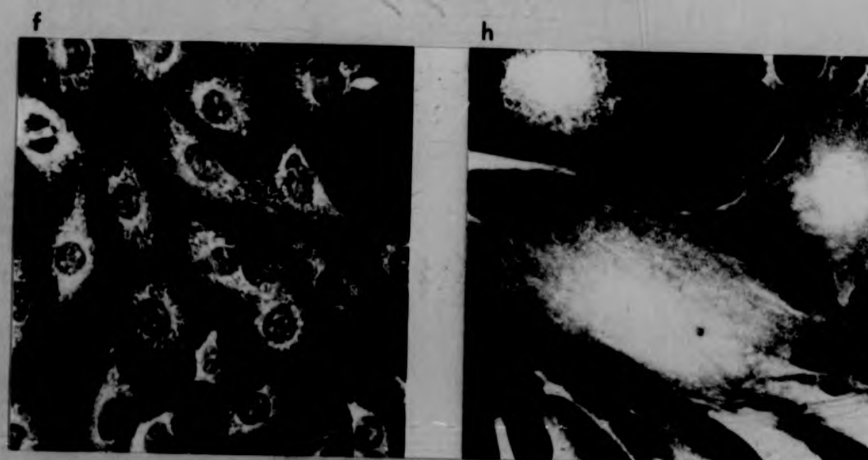
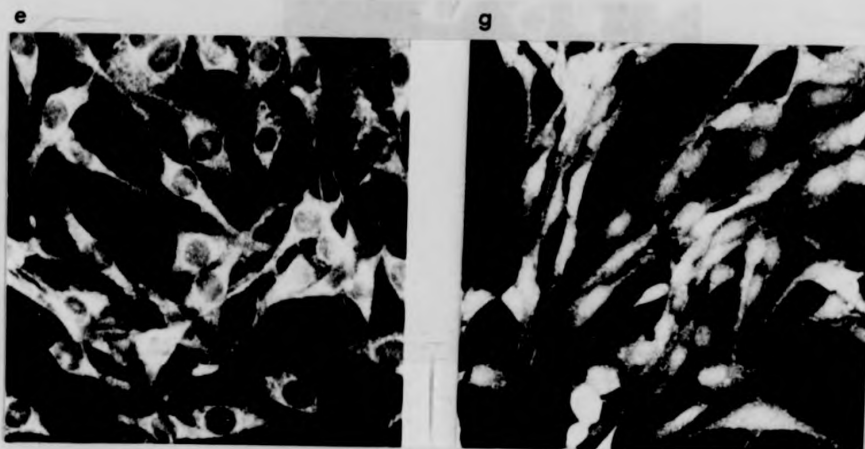


FIGURE 14



e &amp; f MSV C3H2

g &amp; h MSVMEF2

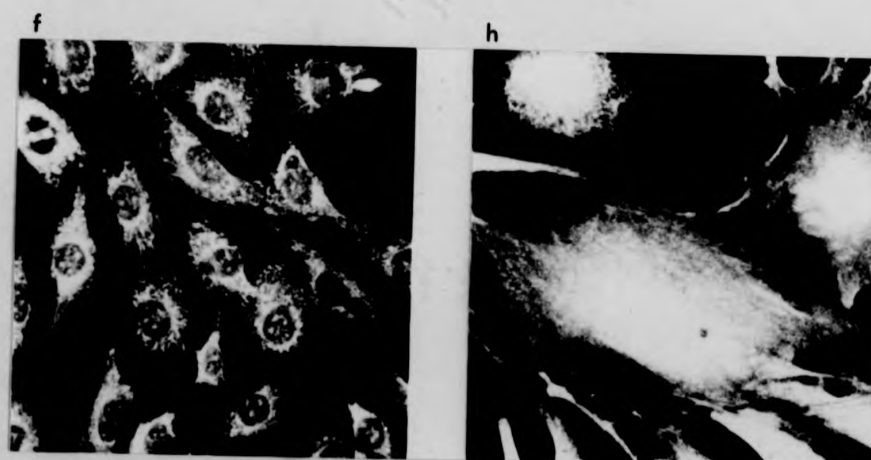
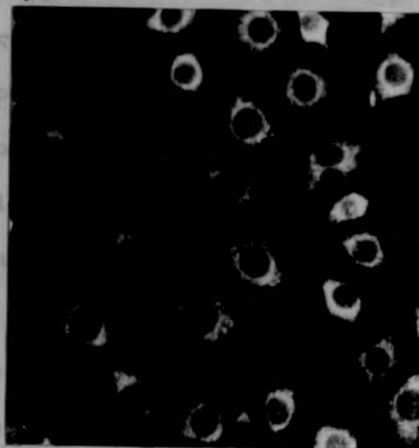


FIGURE 14



MSV C3H6



observed (Fig. 14) . whilst they could be seen in the flattened cells in the presence of interferon, but not in the more rounded cells (Fig. 14).

Microfilaments are prominent in control cultures, but are absent in the cells of interferon-treated cultures.

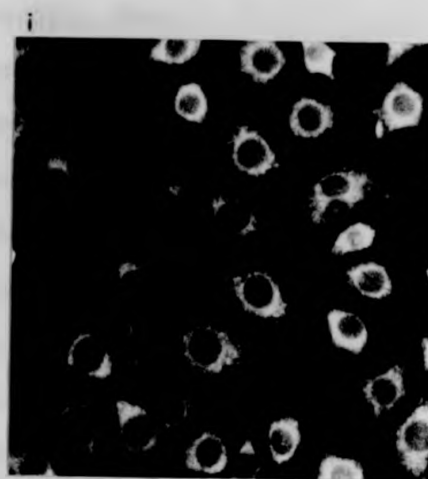
Overall, Figure 14 shows that the cell size following the treatment with interferon is equivocal evidence to the presence of microfilament bundles, and the degree of polymerization of actin.

A method devised by Elikstad et al. (30) which measured the inhibition of  $\text{Mg}^{2+}$  activity by monomeric actin, but not polymerized actin. Unfortunately, this assay could not be used to function reliably and was abandoned.

#### Cell Surface Fibronectin

Since fibronectin is a major component of the extracellular matrix, its presence in normal cells appears to be related to cell adhesion and migration. In transformed cells, it is absent. The transformed phenotype is characterized by a loss of cell adhesion, which is likely since some agents, such as retinoic acid, stimulate the reconstruction of the intracellular microfilament system. The presence of fibronectin is usually paralleled that of surface fibronectin (31, 33).

FIGURE 14



MSV C3H6



interferon (fig. 14g) , whilst they could be seen in the flattened cells in the presence of interferon, but not in the more rounded cells (fig. 14h) .

Microfilaments are just visible in a few MSV C3H6 cells in control cultures, but they are rather more apparent in the cells of interferon-treated cultures .

Overall, figure 14 illustrates clearly the increase in cell size following interferon treatment, but gives rather equivocal evidence to support the reconstitution of the microfilament bundles. Attempts were made to quantitate the degree of polymerisation of actin within cells using a method devised by Blikstad et al. (32) which measured the inhibition of DNase I activity by monomeric actin, but not polymerised actin. Unfortunately this assay could not be made to function reliably and so was abandoned.

#### D) Cell Surface Fibronectin

Since fibronectin is a cell surface protein which in normal cells appears to play a major role in cell spreading and adhesion, but is usually lost or greatly reduced upon transformation, it is possible that any agent that reverses the transformed phenotype might in so doing increase the amount of cell surface fibronectin. This seems all the more likely since some agents, such as cyclic AMP or butyric acid, stimulate the reconstruction of the intracellular microfilament system, the presence of which usually parallels that of surface fibronectin (131, 230) .



In experiments with C3H MEF cells, two distinct patterns were obtained when they were stained with antiserum to fibronectin, depending on how the cells were treated before staining. Fixation and staining of intact confluent cells revealed a complex intercellular branching matrix running across the upper surfaces of the cells (figure 15a). Extraction of live cells with 1% NP40 prior to fixation left a footprint representing the subcellular matrix which when stained with fibronectin antiserum revealed a fine branching meshwork not unlike the microfilament system (figure 16a). These observations are similar to those of Mautner and Hynes (177) who studied the fibronectin distribution on the surfaces of hamster NIL 8 fibroblasts.

Further observations of the matrix of the upper surface of intact C3H10T $\frac{1}{2}$  and transformed cells are shown in figure 15b-d, while the subcellular matrix of NP40-extracted C3H10T $\frac{1}{2}$  cells only is shown in figure 16b. Butyric acid treatment of C3H MEF cells was not recorded, but its treatment of the other cell types was. The layout of photographs in figure 15b-d and figure 16b is the same as that described for figure 13, i.e. for each cell type there are four photographs:-  
i) control, ii) with  $10^4$  units/ml interferon, iii) with 0.5 mM butyric acid, and iv) with 0.5 mM butyric acid and  $10^4$  units/ml interferon.

Confluent C3H10T $\frac{1}{2}$  cells showed two distinct patterns, but in both cases the fibronectin matrix was much less

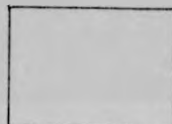


LEGEND TO FIGURE 15

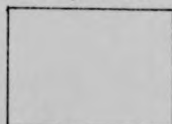
Effects of interferon and butyric acid on fibronectin distribution. Coverslips were fixed in 3.5% formaldehyde in PBS.

Plate a) arranged as follows:-

(i) control



(ii)  $10^4$  U/ml IFN



Plates b-d arranged as follows:-

(i) control



(ii)  $10^4$  U/ml IFN



(iii) 0.5mM BA



(iv)  $10^4$  U/ml IFN  
& 0.5mM BA



Plates: a) C3H MEF; b) C3H 10T $\frac{1}{2}$ ; c) MSV C3H6;  
d) MSV MEF2

Scale:  100um

FIGURE 15a



C3H MEF

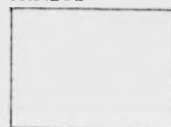


# LEGEND TO FIGURE 15

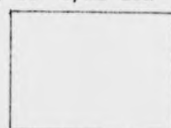
Effects of interferon and butyric acid on fibronectin distribution. Coverslips were fixed in 3.5% formaldehyde in PBS.

Plate a) arranged as follows:-

(i) control



(ii)  $10^4$  U/ml IFN

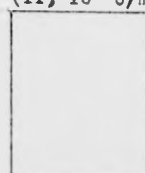


Plates b-d arranged as follows:-

(i) control



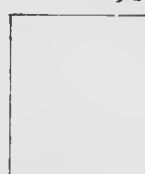
(ii)  $10^4$  U/ml IFN



(iii) 0.5mM BA



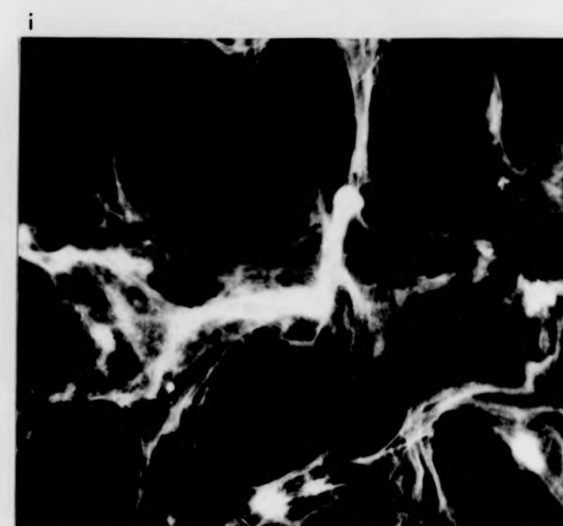
(iv)  $10^4$  U/ml IFN  
& 0.5mM BA



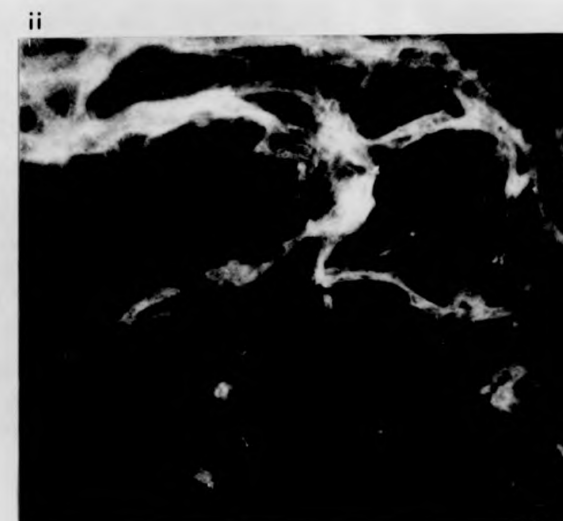
Plates: a) C3H MEF; b) C3H 10T $\frac{1}{2}$ ; c) MSV C3H6;  
d) MSV MEF2

Scale:  100 $\mu$ m

FIGURE 15a



C3H MEF

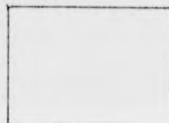


# LEGEND TO FIGURE 15

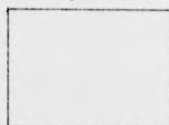
Effects of interferon and butyric acid on fibronectin distribution. Coverslips were fixed in 3.5% formaldehyde in PBS.

Plate a) arranged as follows:-

(i) control

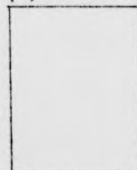


(ii)  $10^4$  U/ml IFN

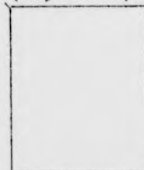


Plates b-d arranged as follows:-

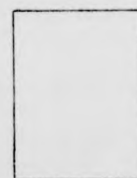
(i) control



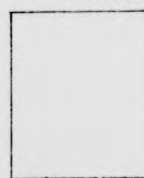
(ii)  $10^4$  U/ml IFN



(iii) 0.5mM BA



(iv)  $10^4$  U/ml IFN  
& 0.5mM BA



Plates: a) C3H MEF; b) C3H 10T $\frac{1}{2}$ ; c) MSV C3H6;  
d) MSV MEF2

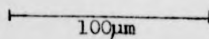
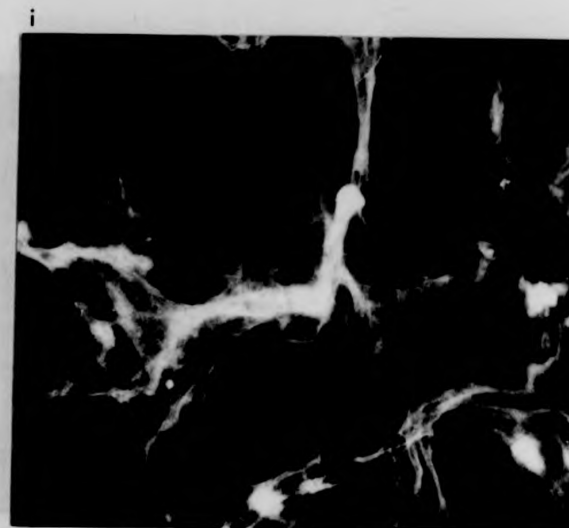
Scale:  100 $\mu$ m

FIGURE 15a



C3H MEF

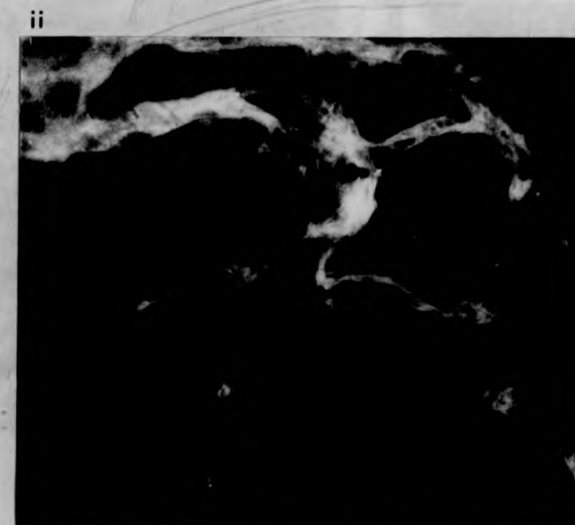


FIGURE 15b

C3H 10T $\frac{1}{2}$ 

FIGURE 15b

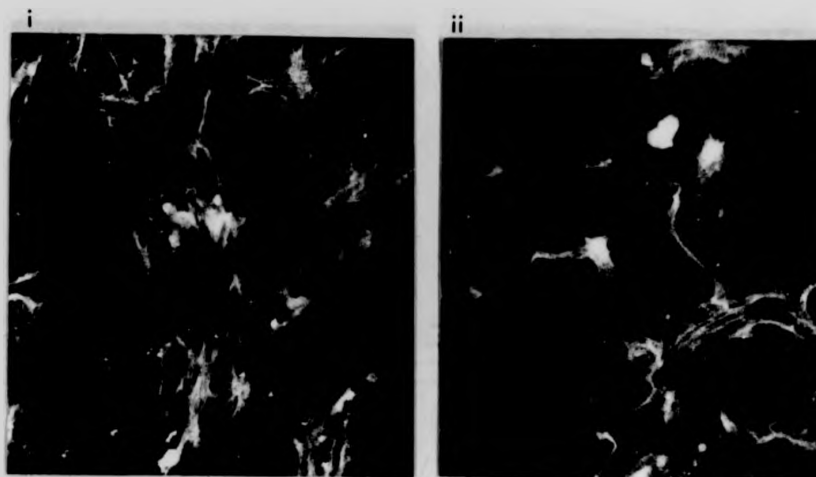
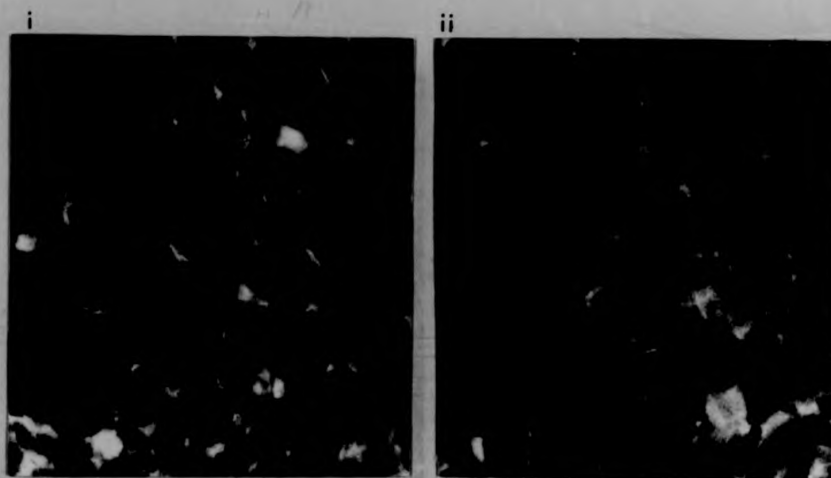
C3H 10T $\frac{1}{2}$ 

FIGURE 15c



MSV C3H6

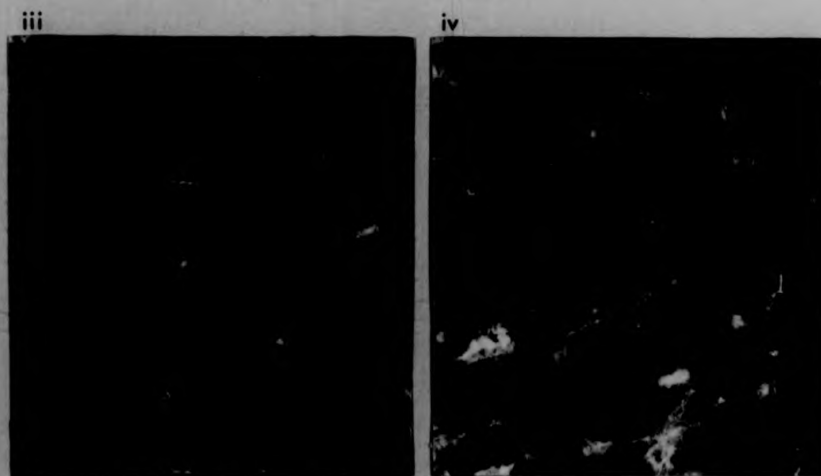
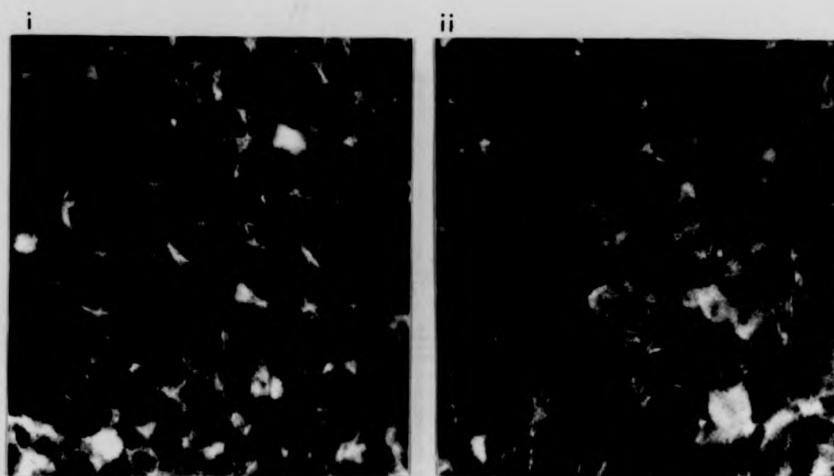




FIGURE 15c



MSV C3H6

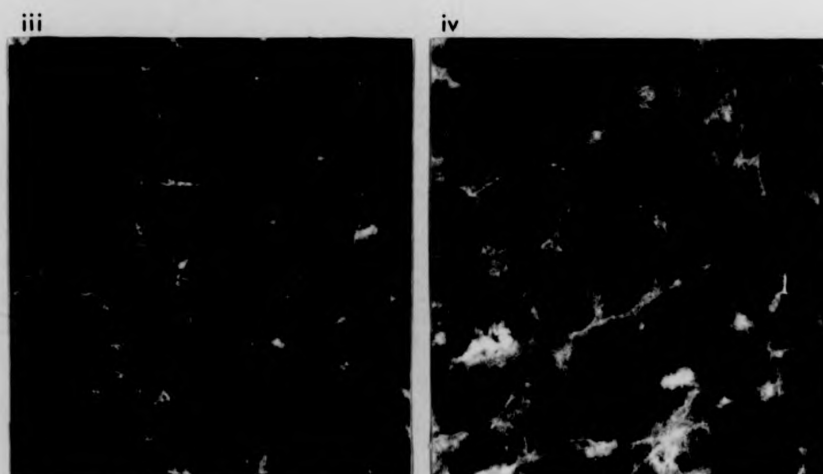
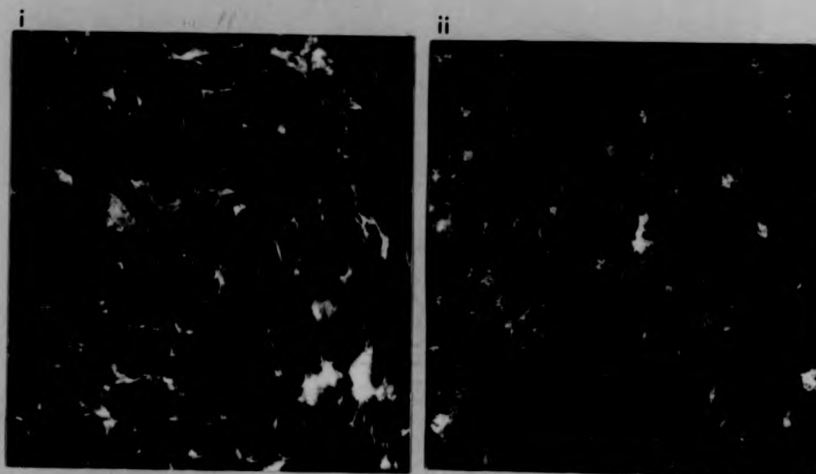


FIGURE 15d



MSV MEF2

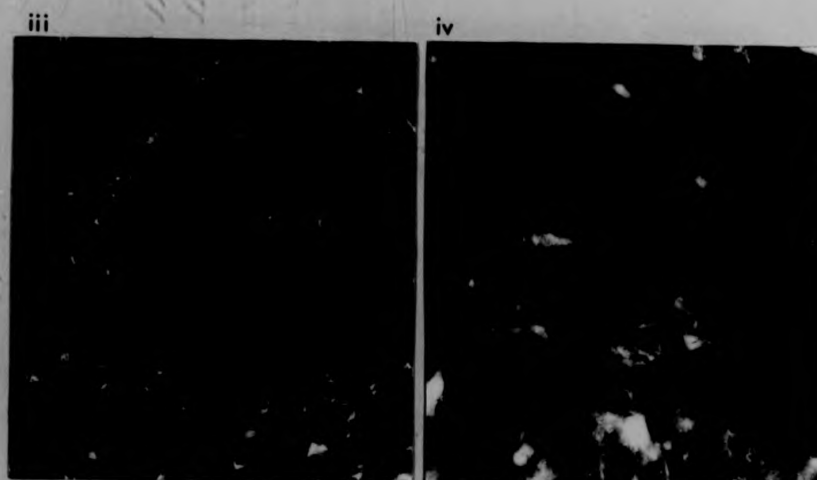
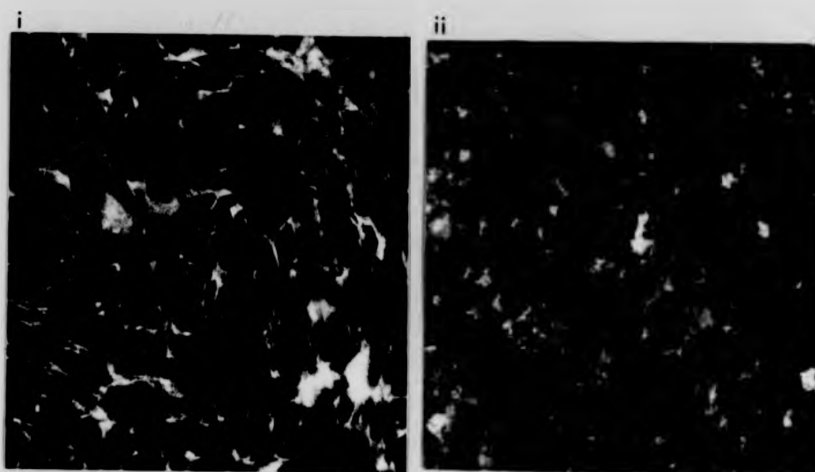




FIGURE 15d



MSV MEF2



LEGEND TO FIGURE 16

Effects of interferon and butyric acid on distribution of fibronectin in subcellular matrix. Cells were grown on glass coverslips, treated with 1% NP40, fixed with 3.5% formaldehyde in PBS and then stained with antiserum to fibronectin.

Plates: a) C3H MEF; b) C3H 10T $\frac{1}{2}$

Treatments: a(i) and b(i) controls;

a(ii) and b(ii)  $10^4$  U/ml IFN.

b(iii) 0.5mM BA

b(iv) 0.5mM BA &  $10^4$  U/ml IFN.

Scale:

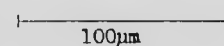


FIGURE 16a



C3H MEF



LEGEND TO FIGURE 16

Effects of interferon and butyric acid on distribution of fibronectin in subcellular matrix. Cells were grown on glass coverslips, treated with 1% NP40, fixed with 3.5% formaldehyde in PBS and then stained with antiserum to fibronectin.

Plates: a) C3H MEF; b) C3H 10T $\frac{1}{2}$

Treatments: a(i) and b(i) controls;

a(ii) and b(ii)  $10^4$  U/ml IFN.

b(iii) 0.5mM BA

b(iv) 0.5mM BA &  $10^4$  U/ml IFN.

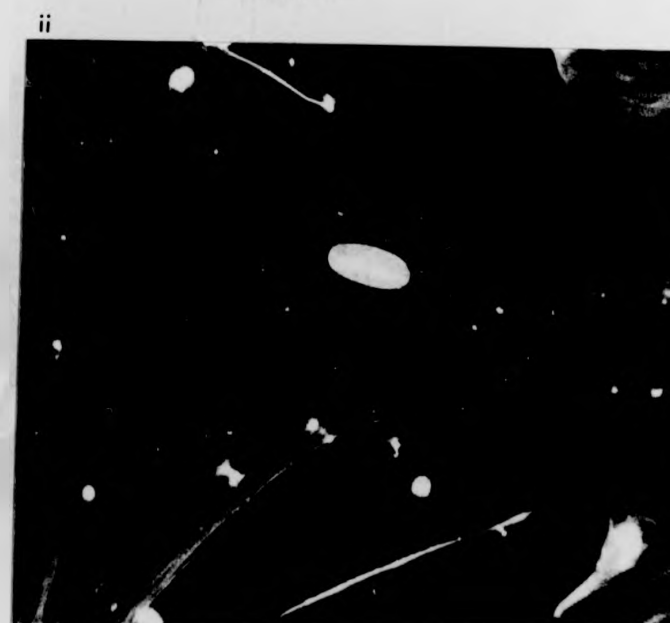
Scale:

100 $\mu$ m

FIGURE 16a



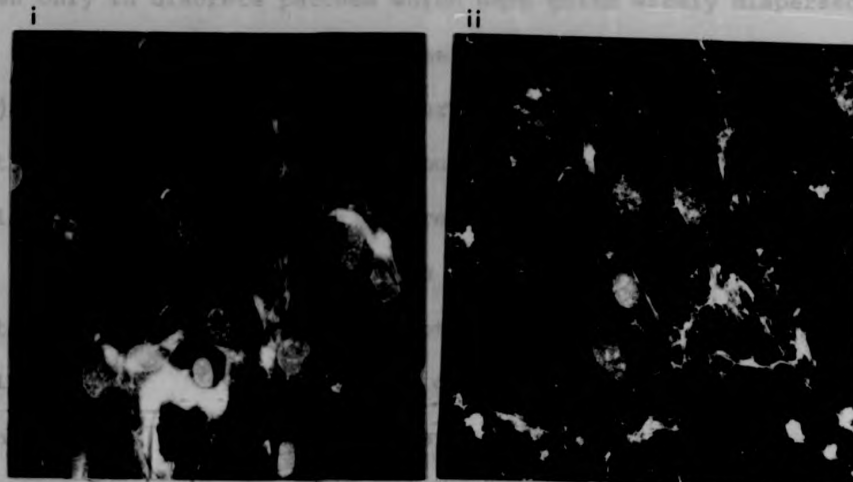
C3H MEF



extensive than on C3H KSF cells, (Figures 15b and 16b).

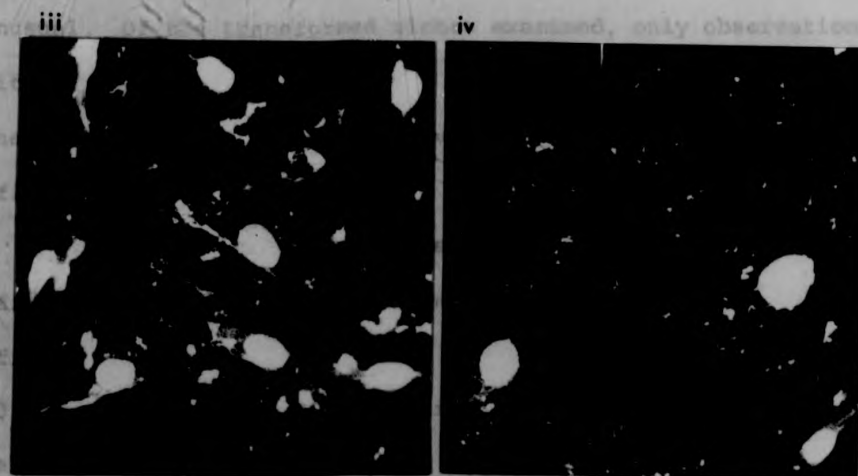
on intact cells (Figure 15b). The present matrix could be seen only in discrete patches which were widely dispersed.

FIGURE 16b



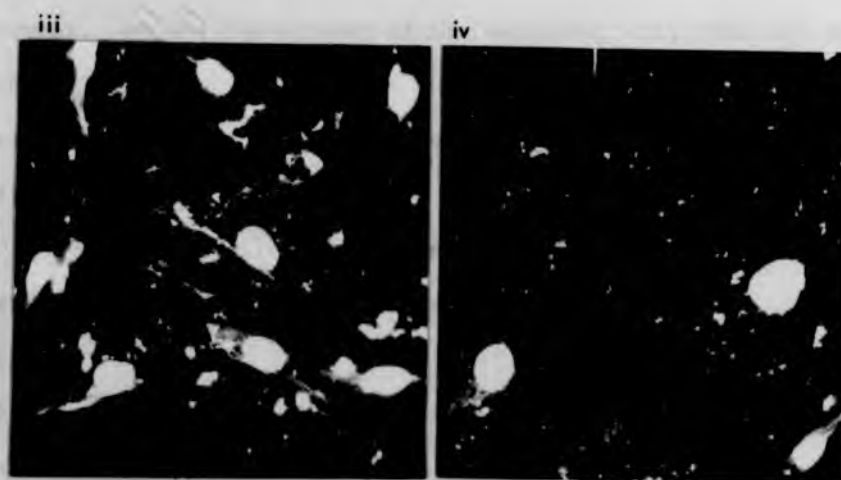
cell-cell adhesion, but only occasionally across the cells' upper surfaces. Though the filopodia present on the surfaces of these transformed cells is clearly reduced when compared with 10T½ cells, the presence of any is quite

C3H 10T½



which became oriented into a filopodial network similar to that seen under C3H KSF cells (Fig. 15a and 15b).

FIGURE 16b

C3H 10T $\frac{1}{2}$ 

extensive than on C3H MEF cells, (figures 15b and 16b). On intact cells (figure 15b) the fluorescent matrix could be seen only in discrete patches which were quite widely dispersed, up to 0.5 mm apart, with occasionally a few fibres connecting adjacent patches. The subcellular matrix seen in NP40 extracted cells (fig. 16b) was found on all cells, but the filamentous network was greatly reduced and less orderly.

The transformed cells showed very little fluorescence after NP40 extraction (results not shown), suggesting poor cell-substrate adhesion. Intact cells showed a matrix of fine fibres which appeared to run between cells, suggesting cell-cell adhesion, but only occasionally across the cells' upper surfaces. Though the fibronectin present on the surfaces of these transformed cells is clearly reduced when compared with C3H10T $\frac{1}{2}$  cells, the presence of any is quite unusual. Of the transformed clones examined, only observations with MSV C3H6 and MSV MEF2 cells are shown in figure 15, since they are representative of observations made on other clones (figure 15c-d).

$10^4$  units/ml interferon appeared to have no obvious effect on the fibronectin pattern of any of the cell types (figures 15a-d (ii) and 16a (ii) and b (ii)). Butyric acid (0.5 mM) did alter the fibronectin distribution in the subcellular matrix of C3H10T $\frac{1}{2}$  cells (fig 16b (iii) and (iv)), which became organised into a fibrillar network similar to that seen under C3H MEF cells (fig. 16a (i) and (ii)).

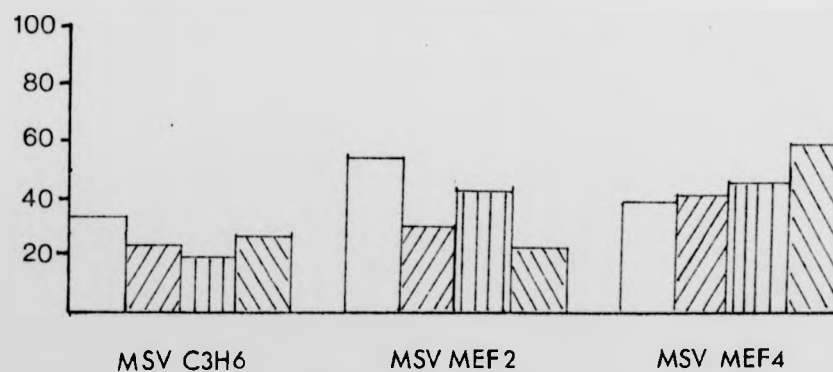
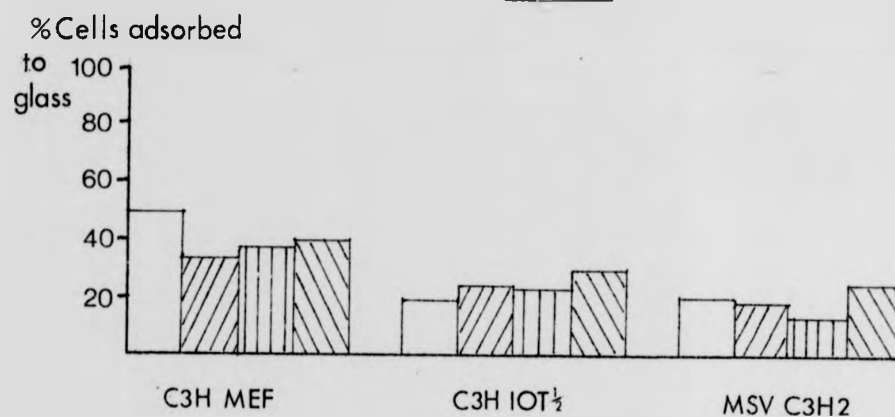
In C3H10T $\frac{1}{2}$  cells butyric acid and interferon together appeared to have no additional action (figs 15b (iv) and 16 (iv) ).

Butyric acid did not alter the fibronectin distribution of any of the transformed clones (fig. 15c (iii) and d (iii) for MSV C3H6 and MSV MEF2 cells respectively). However, butyric acid and interferon together did greatly enhance the intercellular matrix on MSV C3H6 cells (fig. 15c (iv) ). Observations with MSV C3H2 and MSV MEF2 cells were less clear. There may have been an increased fibronectin matrix, but in both clones the intensity and clarity of staining were reduced (see fig. 15d (iv) for MSV MEF2). On the other hand, with MSV MEF4 cells it was quite clear that the fibronectin distribution remained unchanged (results not shown).

#### E) Adhesion to Glass Surfaces

The observations that interferon has little effect on the fibronectin distribution would suggest that either interferon-treated cells are no more or less adherent to the substratum, or fibronectin is not involved in adhesion, contrary to current ideas.

To test this, suspended radioactive cells were allowed to adsorb at 37°C to the base of glass scintillation vials which had previously been soaked with PBS or fibronectin purified from new-born calf serum. Serum contains fibronectin which when in contact with a substratum at 37°C, such as glass, will

FIGURE 17

Cell lines

Ability of Cells Grown in Interferon to Adsorb to GlassKEYCells grown inIFN(10<sup>4</sup>U/ml)

—

—

+

+

Glass vials presoakedwith fibronectin(100ug/ml)

—

+

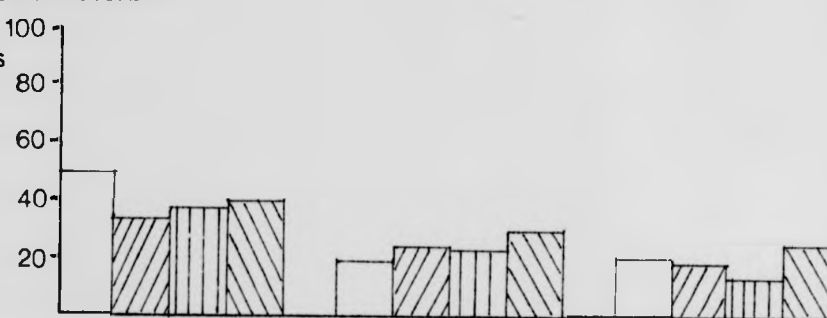
—

+



FIGURE 17

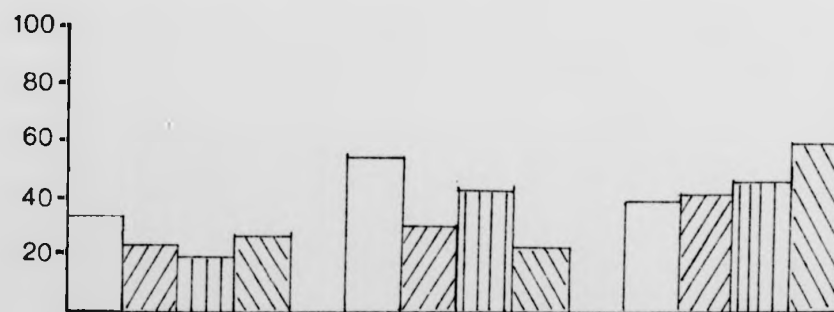
%Cells adsorbed

to  
glass

C3H MEF

C3H 10T<sub>1/2</sub>

MSV C3H2



MSV C3H6

MSV MEF2

MSV MEF4

Cell lines

Ability of Cells Grown in Interferon to Adsorb to GlassKEYCells grown inIFN(10<sup>4</sup>U/ml)

—

—

+

+

Glass vials presoakedwith fibronectin(100ug/ml)

—

+

—

+

attach to it and may subsequently affect the ability of cells to adsorb to the glass (126, 199).

The results in figure 17 show only small and inconsistent changes in cell adsorption which suggest that interferon had little effect on the adhesiveness of cells.

#### F) Polyacrylamide Gel Electrophoresis

The photographs in figures 14 and 15 suggested that interferon alone or in conjunction with butyric acid could alter the intra- and extracellular distributions of actin and fibronectin respectively. Figure 15c (iv) which recorded the effects of butyric acid and interferon together on MSV C3H6 suggested that the total amount of fibronectin may be increased.

To assess whether interferon and butyric acid could alter the total quantities of these proteins cultures were labelled with <sup>35</sup>S-methionine, lysed and resolved on 8% polyacrylamide gels. As can be seen from the gels in figure 18, C3H10T $\frac{1}{2}$  cells possessed considerably more fibronectin than the transformed clones, but little difference could be seen in the levels of total actin. Neither interferon, nor butyric acid treatment had any clear effect on levels of fibronectin or actin, even in MSV C3H6 cells treated with both agents.

Quantitation of these two proteins was attempted by excising the respective bands from the gels, dissolving the proteins in scintillant and then counting the radioactivity.

The histograms in figures 19 and 20 show the counts per

# LEGEND TO FIGURE 18

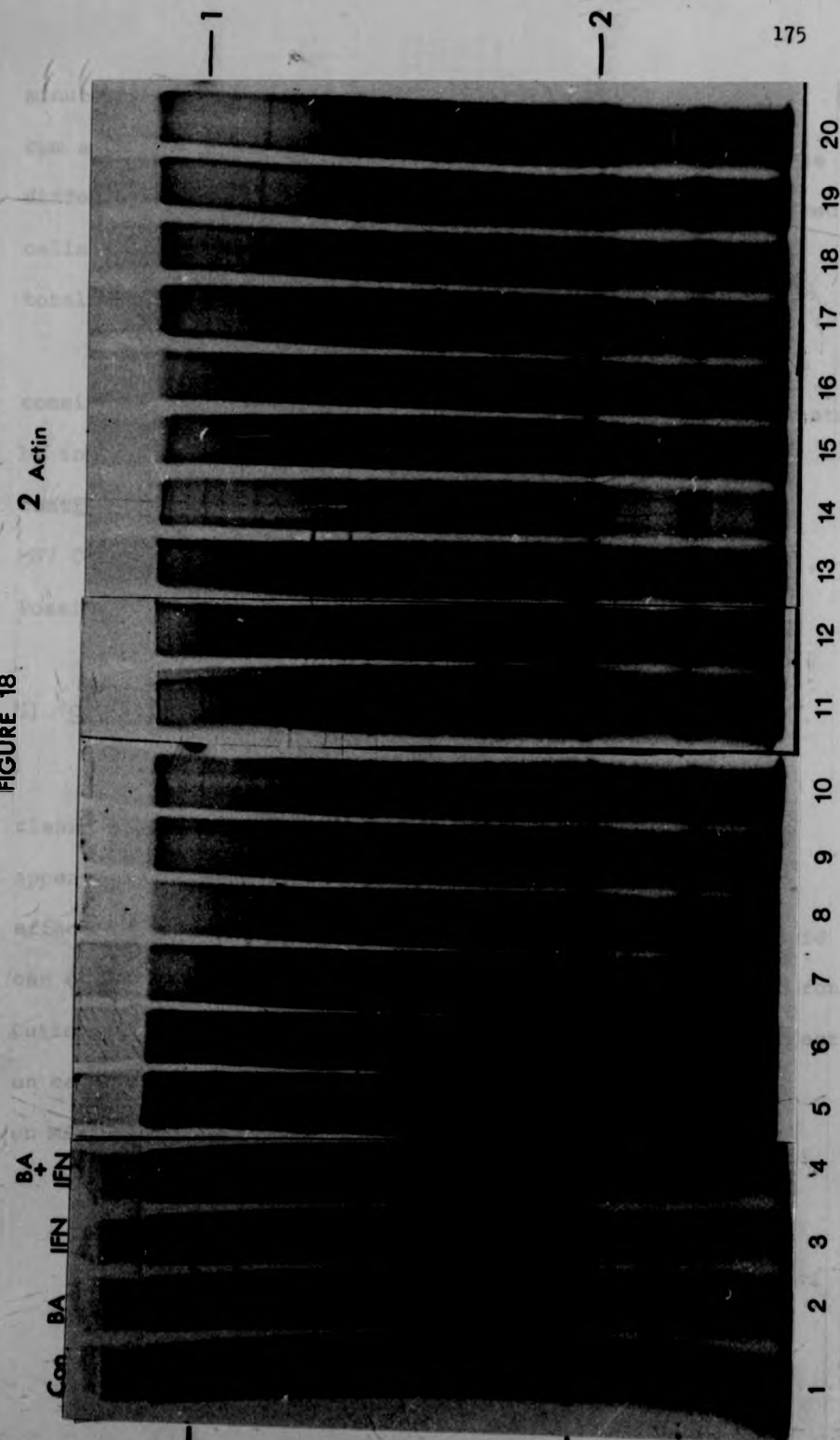
SDS polyacrylamide gel electrophoresis of whole cell lysates taken from cells grown with or without interferon ( $10^4$  U/ml) and/or butyric acid (0.5mM), and labelled with  $^{35}$ S-methionine.

Cells used: C3H 10T $\frac{1}{2}$  (tracks 1 - 4); MSV C3H2 (tracks 5 - 8); MSV MEF2 (tracks 9 - 12); MSV C3H6 (tracks 13 - 16); MSV MEF4 (tracks 17 - 20)

Treatments for each cell type are in the order shown for C3H 10T $\frac{1}{2}$  cells (tracks 1 - 4). Thus;

Tracks; 1, 5, 9, 13, 17 - cells grown without IFN or BA.  
 2, 6, 10, 14, 18 - " " with 0.5mM BA.  
 3, 7, 11, 15, 19 - " "  $10^4$  U/ml IFN.  
 4, 8, 12, 16, 20 - " " " " " & 0.5mM BA.

FIGURE 18  
 1 Fibronectin  
 2 Actin



# LEGEND TO FIGURE 18

SDS polyacrylamide gel electrophoresis of whole cell lysates taken from cells grown with or without interferon ( $10^4$  U/ml) and/or butyric acid (0.5mM), and labelled with  $^{35}$ S-methionine.

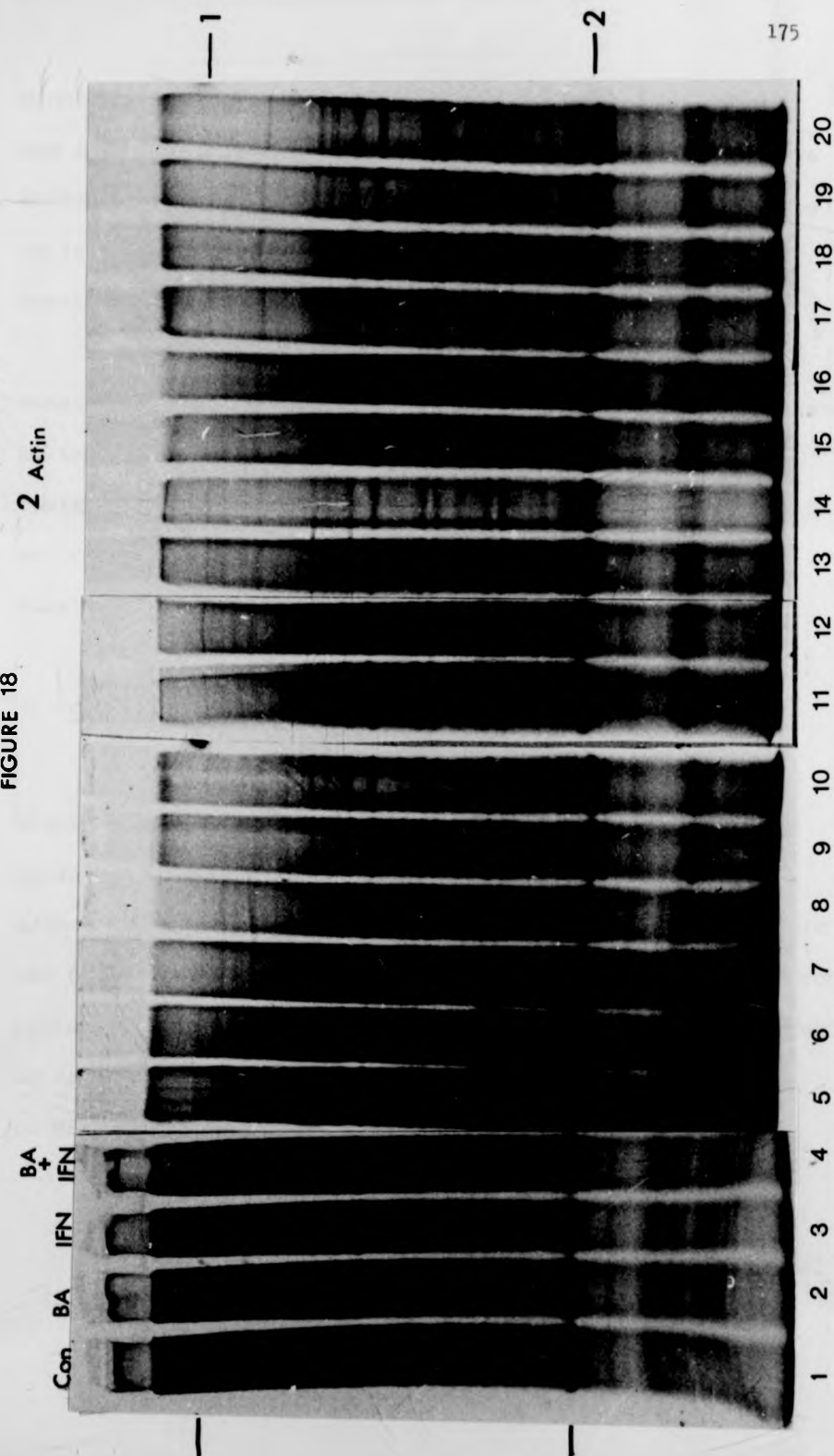
Cells used: C3H 10T $\frac{1}{2}$  (tracks 1 - 4); MSV C3H2 (tracks 5 - 8); MSV MEF2 (tracks 9 - 12); MSV C3H6 (tracks 13 - 16); MSV MEF4 (tracks 17 - 20)

Treatments for each cell type are in the order shown for C3H 10T $\frac{1}{2}$  cells (tracks 1 - 4). Thus;

Tracks: 1, 5, 9, 13, 17 - cells grown without IFN or BA.  
 2, 6, 10, 14, 18 - " " with 0.5mM BA.  
 3, 7, 11, 15, 19 - " "  $10^4$  U/ml IFN.  
 4, 8, 12, 16, 20 - " " " " & 0.5mM BA.

1 Fibronectin  
 2 Actin

FIGURE 18



minute in each band represented as a percentage of the total cpm applied to each track. They show quite clearly the large difference in fibronectin possessed by C3H10T $\frac{1}{2}$  and transformed cells (fig. 20) and also a two to five fold difference in total actin is revealed (fig. 19).

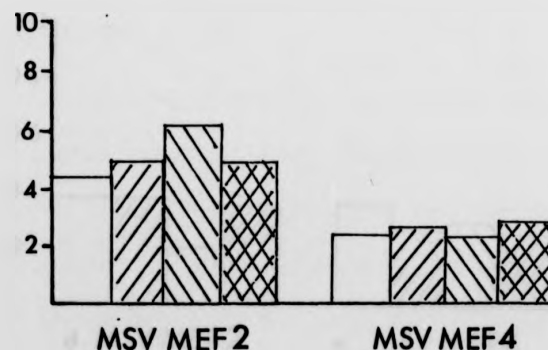
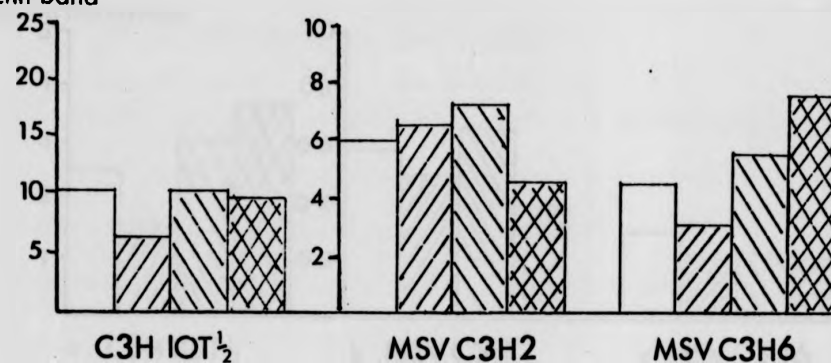
These histograms also demonstrate quite clearly that no consistent changes in actin or fibronectin levels were initiated by interferon or butyric acid treatment. This obviously contradicts the observed increase in fibronectin matrix in MSV C3H6 cells treated with interferon and butyric acid. Possible explanations will be put forward in the Discussion.

#### G) Conclusions

The data presented in this chapter show that interferon clearly gives transformed cells a more normal morphology, appears to restore the microfilament system, but has little effect on cell surface fibronectin distribution. Butyric acid can cause further flattening when added jointly with interferon. Curiously, either treatment alone appears to have little effect on cell surface fibronectin, but the two together, at least on MSV C3H6 cells, greatly increase its organisation.

FIGURE 19

% cpm per gel present  
in actin band



Levels of Actin in Cells Treated with Butyric Acid and  
Interferon for Upto One Week

Key for Figs 19 & 20



O IFN; O BA



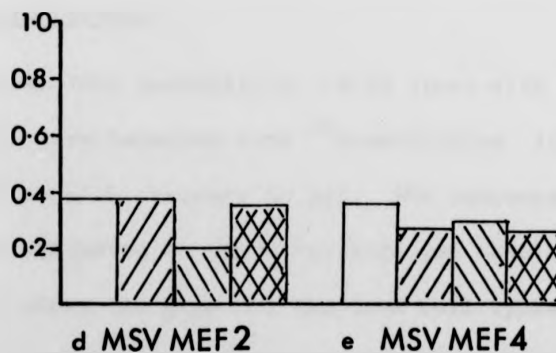
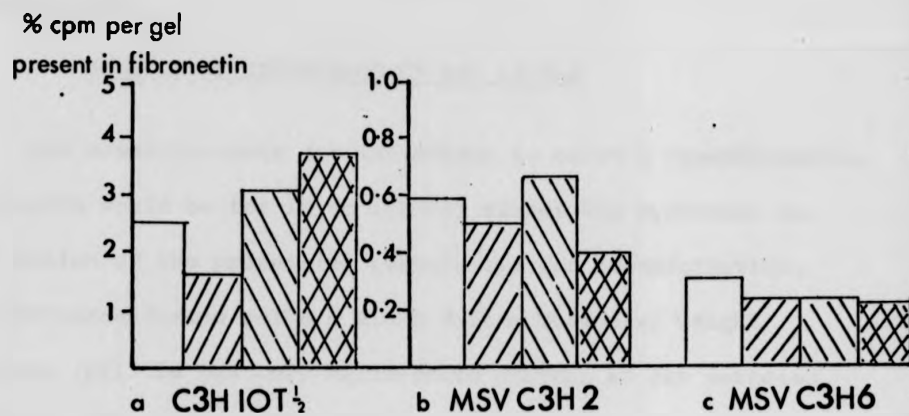
O IFN; 0.5mM BA



$10^4$  U/ml IFN; O BA



$10^4$  U/ml IFN; 0.5mM BA

FIGURE 20

Levels of Fibronectin in Cells Treated with Butyric Acid and Interferon

Key - As for Fig 19



## CHAPTER FIVE

### EFFECTS OF INTERFERON ON p21 LEVELS

One possible route for interferon to exert a transformation reversion would be for it to inhibit either the synthesis or the action of the protein(s) responsible for transformation. In KiSV-transformed cells a 21000 dalton molecular weight protein (p21) is the only virus-coded protein so far detected, and it may be this protein which is responsible for transformation (129, 227, 228).

#### A) Electrophoresis

To test this possibility, cells grown with or without interferon were labelled with  $^{35}\text{S}$ -methionine, lysed and immunoprecipitated with antisera to p21. The immunoprecipitates were then subjected to polyacrylamide gel electrophoresis. Figure 21 shows the gels for the five cell types examined.

No p21 could be detected in C3H10T $\frac{1}{2}$  cells (gel 1, tracks 1-4), but all the transformed cells contained quantities which could be seen when immunoprecipitated, but not when treated with control sera (gel 1 tracks 2, 4, 6, 8, 10, 12; gel 2 tracks 2, 4, 6, 8). The densities of the p21 bands suggest that interferon treatment did decrease the amounts of p21 present, but since the interferon-treated samples incorporated generally less  $^{35}\text{S}$ -methionine than controls, this may not be a real change.



# LEGEND TO FIGURE 21

SDS polyacrylamide gel electrophoresis of cell lysates precipitated with antisera to p21. Cells grown with or without interferon ( $10^4$  U/ml) and labelled with  $^{35}$ S-methionine before lysis and immunoprecipitation.

## Gel 1:

Tracks: 1 - 4 C3H 10T $\frac{1}{2}$ ; 5 - 8 MSV C3H2; 9 - 12 MSV C3H6;

Treatments: 1, 2, 5, 6, 9, 10 - cells grown with  $10^4$  U/ml IFN.  
3, 4, 7, 8, 11, 12 - cells grown with no IFN.

## Immunoprecipitation:

2, 4, 6, 8, 10, 12 - cell lysates treated with control sera.

1, 3, 5, 7, 9, 11 - cell lysates treated with anti-p21 sera.

## Gel 2:

Tracks; 1 - 4 MSV MEF 2; 5 - 8 MSV MEF4.

Treatments: 1, 2, 5, 6 - cells grown with  $10^4$  U/ml IFN.  
3, 4, 5, 8 - " " " no interferon.

## Immunoprecipitation:

2, 4, 6, 8 - cell lysates treated with control sera.

1, 3, 5, 7 - " " " " anti-p21 sera.

Bands labelled 1, 2, 3 are the proteins which were cut out and cpm determined for comparison with cpm of p21 in samples from interferon-treated and control cultures.

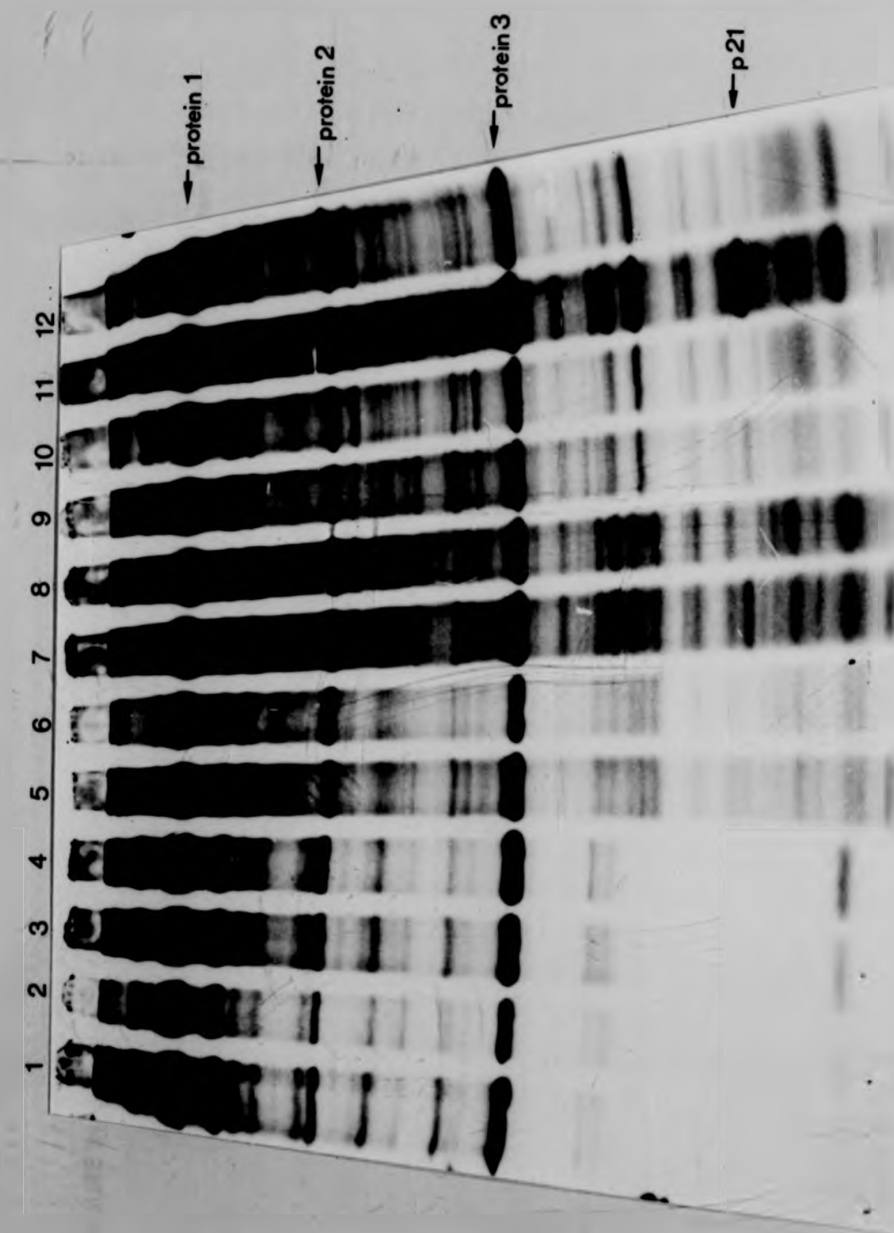


FIGURE 21

GEL 1

# LEGEND TO FIGURE 21

SDS polyacrylamide gel electrophoresis of cell lysates precipitated with antisera to p21. Cells grown with or without interferon ( $10^4$  U/ml) and labelled with  $^{35}$ S-methionine before lysis and immunoprecipitation.

## Gel 1:

Tracks: 1 - 4 C3H 10T; 5 - 8 MSV C3H2; 9 - 12 MSV C3H6;

Treatments: 1, 2, 5, 6, 9, 10 - cells grown with  $10^4$  U/ml IFN.  
3, 4, 7, 8, 11, 12 - cells grown with no IFN.

## Immunoprecipitation:

2, 4, 6, 8, 10, 12 - cell lysates treated with control sera.

1, 3, 5, 7, 9, 11 - cell lysates treated with anti-p21 sera.

## Gel 2:

Tracks: 1 - 4 MSV MEF 2; 5 - 8 MSV MEF4.

Treatments: 1, 2, 5, 6 - cells grown with  $10^4$  U/ml IFN.  
3, 4, 5, 8 - " " " no interferon.

## Immunoprecipitation:

2, 4, 6, 8 - cell lysates treated with control sera.

1, 3, 5, 7 - " " " " anti-p21 sera.

Bands labelled 1, 2, 3 are the proteins which were cut out and cpm determined for comparison with cpm of p21 in samples from interferon-treated and control cultures.

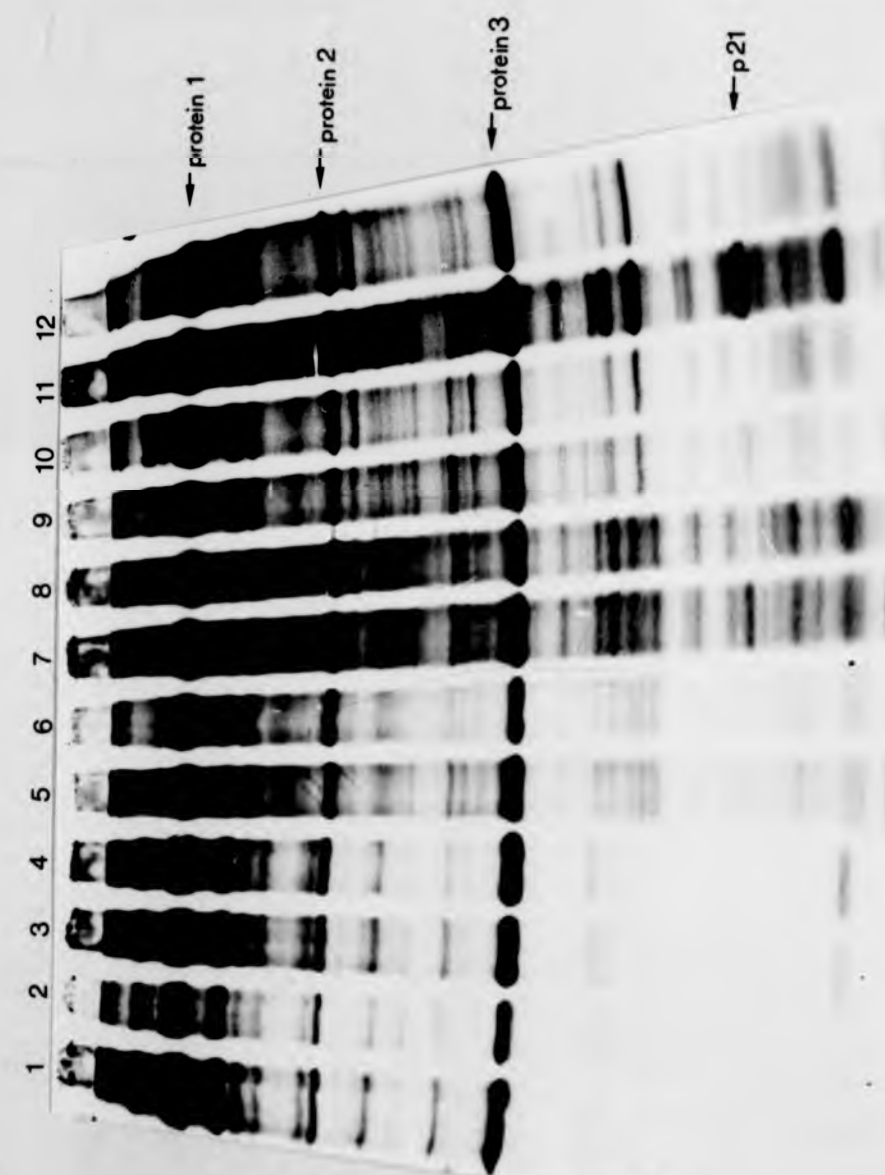


FIGURE 21

GEL 1

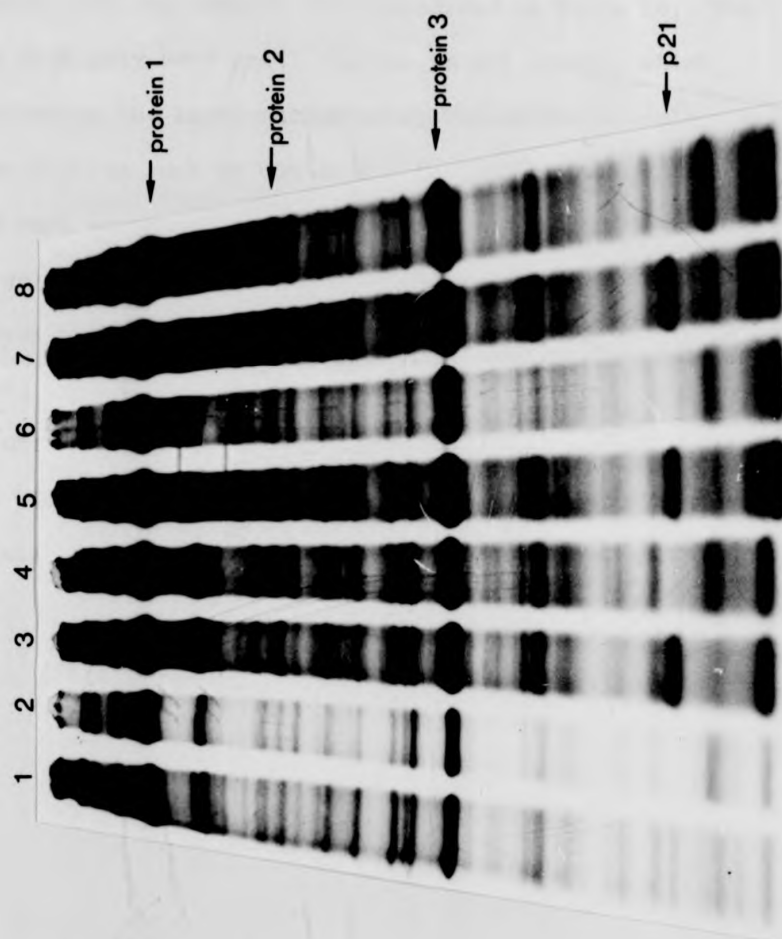


FIGURE 21

GEL 2

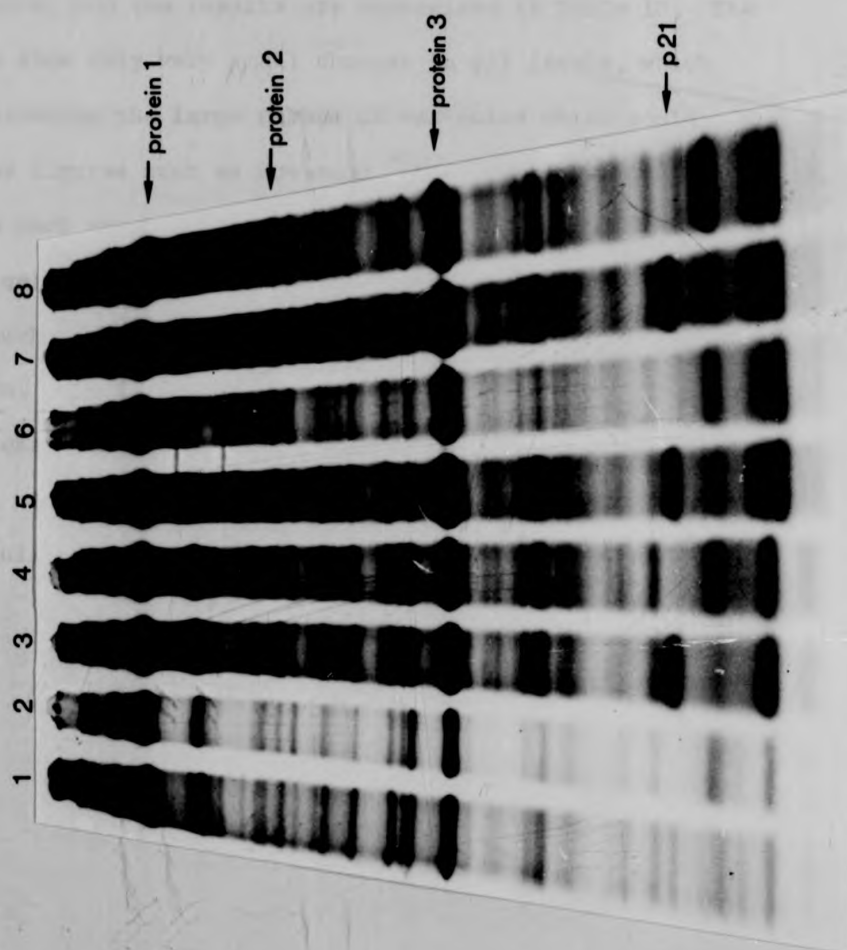


FIGURE 21

GEL 2

### B) Quantitation

Quantitation was attempted as described in Materials and Methods, and the results are summarised in Table 10. The data show only very small changes in p21 levels, which, considering the large number of variables which could affect these figures such as how much  $^{35}\text{S}$ -methionine was incorporated into each sample, the efficiency of extraction of protein from the gel to measure the cpm in a single protein and the accuracy or such a procedure, do not permit firm conclusions to be drawn. However, it is interesting that the relative size of the change between the different clones corresponds fairly well with their observed sensitivities towards interferon's cellular activities.

TABLE 10

Cell Line	Change in p21 Levels in IFN- Treated Cells Relative to Levels in Controls, as measured by -		
	Total Protein (Lowry)	Total cpm	Relative to proteins 1, 2, 3.
MSV C3H2	-3x	-4x	-2x
MSV C3H6	-6x	-6x	-4x
MSV MEF2	-2x	-2x	-2x
MSV MEF4	+2x	+2x	+2x

Comparison of p21 Levels in Interferon-Treated and Control  
Cell Cultures

+/- indicates that interferon treatment resulted in  
an increase in p21 levels.

-/- indicates that interferon treatment resulted in  
a decrease in p21 levels.

x/- means 'fold change'.

Thus '-3x' means 'a three fold reduction' in levels  
of p21.

## DISCUSSION

This Discussion will be divided into several sections. The first will consider the quality of the interferon used, as regards its suitability, purity and doses used in these studies in relation to interferon preparations used by others. The second section will consider in sequence the experiments described in chapters 2 to 5 to assess the conclusions that may be drawn from the results obtained and how such conclusions may be related to data that have been obtained by others. The final section will summarise these conclusions to give an overall view. The significance of the conclusions and how closely they fulfil the aims of this thesis will also be assessed, and some proposals for further work will be given.

### I. Use of Interferon

#### 1. Interferon Purity

Recent studies with pure interferon (58, 82, 134) have shown that the inhibition of cell growth is a property of interferon itself, and not some contaminant in the impure material often used. The majority of studies still use impure interferon, and this study is no exception. The experiments described in chapter one succeeded in raising the specific activity of purified interferon to  $2 \times 10^8$  units/mg protein, still only 10% pure, but the two-step procedure



employed was quick, simple and gave high yields. To raise the specific activity further would have required further purification steps, probably resulting in low final yields, which would have severely limited the supply of interferon for studies of its cell growth and morphological effects. Thus, higher purity was sacrificed in order to ensure large yields of semi-pure interferon. Use of an antibody-agarose column, such as that described by De Maeyer-Guignard et al. (58) may have greatly improved purity. However, no antiserum was available, and it did not seem appropriate to commit large amounts of interferon to attempting to raise antisera.

The specific activity of interferon used in studies on cell growth and morphology varied between  $5 \times 10^7$  and  $2 \times 10^8$  units/mg protein, which compared very favourably with the activities of interferons employed by others. However, without pure interferon it is not possible to be absolutely certain that observed effects are not due to contaminants, even though cell growth inhibition is known to be a property of pure interferon. Confidence that other observations, such as reduced saturation density and morphological effects, were due to interferon is justified by its stability even after pH 2 treatment and inactivity on heterologous species.

## 2. Interferon Doses

One of the most striking features of the studies reported over the past few years is the vast range of interferon doses



used, anything from less than 10 units/ml to  $10^4$  units/ml. The majority of studies have been on the growth inhibitory activity, and most have indicated that maximal inhibition occurs at interferon doses of 100-1000 units/ml (43, 86, 201), considerably lower than the dose for maximum inhibition seen here. It is clear, therefore, that cell types used in many other studies were much more sensitive to interferon than the cells used by myself (5, 82, 119, 143, 201). One human lymphoblastoid line, Daudi, has been shown to be sensitive to as little as 1 unit/ml (5).

Of the cell types used in this study, DNA synthesis in some (MSV C3H2 and MSV MEF 4) was reduced a little by 100 units/ml (see Figure 12) after 24 hours treatment, while from dose-response curves in Figure 6, the doses for half maximal inhibition after 72 hours of interferon treatment (Table 5) suggest that growth of all cell types tested was sensitive to 100 units/ml. These latter data are, however, difficult to interpret due to the additional complication of reduced cell numbers in interferon-treated cultures. This will be discussed in greater detail later.

The effects of interferon on cloning of single cells on plastic illustrate very clearly the great differences in sensitivity between the cell types used here and those used in other experiments. The data presented in Table 7 show that cloning was inhibited by at the most three fold (C3H10T $\frac{1}{2}$ ), while some actually apparently showed a small increase in

cloning efficiency (MSV C3H 2 and NIH 3T3). This should be contrasted with Glasgow et al. (86), for instance, where 300 units/ml greatly reduced cloning efficiency of murine osteogenic sarcoma cells, and  $3 \times 10^3$  to  $3 \times 10^4$  units/ml completely abolished cloning. Similarly O'Shaughnessey et al. (194) found that 1 unit/ml inhibited by 50% cloning of L cells, while 100 units/ml caused 90% inhibition, and Buffett et al. (43) achieved 90% inhibition of cloning of BALB/c 3T3 cells with  $10^4$  units/ml.

## II. The Effects of Interferon

### 1. Antiviral and Cell Growth Inhibitory Activities

a) Interpretation of results The dose-response curves for cell growth inhibition, shown in figure 6, suggest that a three day treatment with interferon at high doses quite considerably inhibited DNA synthesis, an observation which clearly conflicts with findings made later (Table 6), which indicated that a 24 hour interferon treatment of cells at different densities had little effect on growth rate of C3H10T $\frac{1}{2}$  cells at all densities tested, and only affected transformed cells when at high density. This apparent conflict is probably due to a combination of two factors - i) a 24 hour treatment may not exert maximal inhibition of DNA synthesis, and ii) inhibition of  $^3\text{H}$ -thymidine incorporation following a three day interferon treatment is probably

due not just to inhibition of DNA synthesis but also to reduced cell numbers. The data in Figure 7 show that interferon-treated transformed cells may grow to a much lower saturation density than controls. It seems quite likely that after the three day incubation period, cells grown in the presence of large doses of interferon had reached that saturation density and stopped growing, while those with little or no interferon continued to grow rapidly. This would have been reflected by the large dose response curves seen in Figure 6. It also explains why a 24 hour interferon treatment only inhibited growth of transformed cells seeded at high density.

Furthermore, unpublished data of Dr. Alan Morris showed very shallow dose-response curves for a range of murine cell lines that had been treated with interferon for just 24 hours, the maximum observed inhibition being less than 20%. This adds further support to the above suggestions that interferon, after only 24 hour treatment, actually had only a small effect on DNA synthesis.

b) Experimental techniques The above discussion centred solely on assessing whether the method used to measure cell growth inhibition by interferon was actually valid for the question under study. To study interferon's antiviral activity a routine assay procedure was employed which is known to give an accurate measure of a preparation's potency, so its validity has already been established (286). The

principal aim of these experiments, however, was to compare interferon's antiviral and cell growth inhibitory activities. For this to be possible, the two assays should use similar techniques so that cells are grown, receive interferon and its affect assessed under similar conditions. However, in these experiments there were some differences. For instance antiviral activity was measured with cells grown in glass vial inserts with interferon treatment, virus infection and  $^3\text{H}$ -uridine labelling conducted in medium containing only 2% new-born calf serum - standard procedure for our interferon assays on L cells. Cell growth inhibition was measured in microtitre trays using medium containing 10% new-born calf serum throughout. More reliable, comparable results would have been obtained if the procedures for the two measurements had been standardised.

## 2. Growth and Saturation Densities

a) Cell division and DNA synthesis The growth curves in Figure 7 show quite clearly that normal and transformed cells responded quite differently to interferon. The imposition of quite a low saturation density on some transformed clones suggests the reacquisition of density dependent growth control by these cells. However,  $^3\text{H}$ -thymidine labelling data in Table 6 suggest that even in such density-inhibited cells the level of DNA synthesis remained quite high. This could be due to several reasons - (i) even at the highest cell

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density in Table 6 the interferon-imposed saturation density had not been reached; (ii) the plateau in cell numbers in Figure 7 was not due to density dependent growth inhibition, but due to establishment of an equilibrium between growth of new cells and shedding of necrotic cells into the medium; (iii) cell division may have been inhibited but DNA synthesis continued, resulting in polyploid and multinucleate cells; (iv) interferon had less effect on cells seeded at high than those seeded at low density; (v) a 24 hour interferon treatment may be insufficient time to cause maximal inhibition of DNA synthesis.

The first possibility may be responsible but is unlikely since the highest density recorded in Table 6 ( $40000 \text{ cells/cm}^2$ ) was higher than the saturation densities of any of the interferon-treated transformed clones recorded in Figure 7.

The second possibility also seems unlikely since dead cells were not seen in the medium.

There is experimental evidence to support the possibility that both (iii) and (iv) may be involved. Support for continued DNA synthesis but inhibited division has been produced by Pfeffer et al. (201) who found that normal human fibroblasts treated with beta interferon increased in volume, surface area, protein and DNA content, but cell division ceased. In addition the number of binucleate cells increased five-fold. However, there are no reports which have extended these observations to transformed mouse cells.

Evidence that cell growth may become less sensitive to interferon as cell density increased was produced by Kuwata et al. (151) and Gresser et al. (100) who used human RSb cells and mouse L1210 cells respectively. Horoszewicz et al. (121) suggested that this may be related more to growth rate of the cells at the time of interferon addition, rather than density; i.e. fresh sparsely-seeded stationary cells being more sensitive than rapidly growing dense cells. Thus, a 24 hour interferon treatment may have been less effective at shutting off DNA synthesis in transformed cells seeded at  $4 \times 10^4$  cells/cm<sup>2</sup>, than in cells that had grown up to that density in the constant presence of interferon. This could be tested by trypsinising cells that had grown to their maximum density in the presence of interferon, and then reseeding these and control cells, in wells of microtitre trays at  $4 \times 10^4$  cells/cm<sup>2</sup> in the presence or absence of interferon for 24 hours before feeding with <sup>3</sup>H-thymidine.

Though it is possible that (iii) and (iv) may be partially responsible for there appearing to be a high DNA synthesis rate in transformed cells, whose growth has been density inhibited due to interferon treatment, the most probable explanation is simply that interferon takes more than 24 hours to exert its maximal effect on DNA synthesis. Thus 48 or 72 hours may be required to completely halt DNA synthesis in cells seeded at  $4 \times 10^4$  cells/cm<sup>2</sup>. Certainly, in the morphological studies observed changes in cells shape were much

greater after 72 hours treatment than just 24 hours. Due to the effect that interferon has on cell numbers when treatment is continued for several days, it would be difficult to test whether interferon does take several days to exert maximal inhibition of DNA synthesis, except by the use of a chemostat. In this the cell concentration could be maintained at a constant level so that  $^3\text{H}$ -thymidine incorporation would be a true measure of the rate of DNA synthesis.

b) Comparison with other studies on saturation density The reductions in saturation density observed by various workers appear to have varied greatly depending on cell type used, interferon dose, length of experiment and whether or not the medium was changed during the experiment. Many early studies used suspension cells (especially L1210 and L929 cells), which do not possess a 'saturation density' in the sense of density dependent inhibition of growth. Their growth can be propagated indefinitely if the cells are regularly diluted with fresh medium. However, if the medium is not replenished and the cells grow to a high concentration, after a few days growth will halt and cell numbers start to decline. Initial studies on interferon's growth inhibitory activity were conducted without the addition of fresh medium and thus were able to continue for only 3 to 4 days. Macieira-Coelho et al. (172) found that suspension L1210 control cells increased in density by about ten fold over three days and then started to decline, whilst interferon-treated cultures grew to half



this density in four days before starting a similar decline. Had the medium been changed on the third day, both control and interferon-treated cultures may have continued growing. Paucker et al. (198) found that continuous interferon treatment, under conditions where cells were diluted in fresh medium to a density of  $10^5$ /ml every 2-3 days, resulted in complete inhibition of growth after 13 days.

Similarly, experiments with monolayer cultures have been conducted for a week or more with no media changes (151, 170, 190). My experience showed that growth of sparsely seeded cells was restricted after three to four days unless the medium was replenished, and with transformed cells which had reached  $10^5$  cells/cm<sup>2</sup> or more, the medium had to be renewed every day. In my experiments the medium was, therefore, changed every day to ensure that growth was not nutrient limited.

The data presented in Figure 7 show that both the growth rates and the maximum densities of transformed cells and those normal cells sensitive to interferon could be reduced by interferon treatment. The growth curves for MSV MEF2, MSV C3H6 and MSV C3H2 in late passage are similar to those presented by Knight (143) for SV40 and polyoma virus-transformed 3T3 cells. Furthermore the growth curves for NIH MEF and NIH 3T3 4E cells are very similar to Knight's curves for embryonic and 3T3 fibroblasts, showing them to be quite insensitive relative to the transformed cells. However, the

data with C3H MEF and C3H10T $\frac{1}{2}$  cells agree more closely with work by others, such as Pfeffer et al. (201), Lindahl-Magnusson et al. (166) and Ohwaki and Kawade (190), that growth rate of normal cells can be greatly slowed.

The experiments by Tovey and Brouty-Boye (254) which showed that the viability of L1210 cells grown in a chemostat decreased at low growth rates and that interferon's growth inhibition could thus cause cell death may be a possible explanation for the very slow growth seen in interferon-treated C3H MEF and C3H10T $\frac{1}{2}$  cultures. As already discussed this seems unlikely since cell debris was not observed in the media of such cultures, though because viabilities were not determined this cannot be definitely resolved. However, the majority of studies on growth inhibition in batch cultures have found that interferon does not decrease cell viability (54, 100, 143, 166, 190).

c) Role of serum concentration Clearly serum levels had a profound effect on cells' responses to interferon, 50% serum virtually eliminating the growth inhibition and greatly reducing the effect on saturation density in C3H10T $\frac{1}{2}$  and MSV C3H6 cells (Figure 10). This raised the problem of what serum level is appropriate for these experiments, especially when one considers that in vivo serum levels are very high.

Whether inhibition of interferon action by 50% serum was general for all the clones was not tested, but clearly at 10% serum the different nontransformed types and transformed

clones varied greatly in their sensitivity towards interferon, both as regards inhibition of growth rate and reduction of saturation density. This variation may be related to differences in serum dependence of the different cell types, though this was not tested.

Several reports have suggested that growth inhibition by interferon may be exerted by making cells more sensitive to an existing growth control mechanism (121, 172, 201). That this control mechanism may be serum-linked is supported by these observations and similar studies by Gresser et al. (100). Pfeffer et al. (201), on the other hand, found that serum concentrations in the range 5-20% had no effect on interferon's inhibition of growth of human fibroblasts, which concurs with my findings for cloning of single cells in liquid medium (Table 8), though they contradict those of Kading et al. (138). This possible growth control mechanism, and its relationship to transformation is discussed in more detail later.

d) Passage level The growth curves of Figure 7 showed that the growth of MSV-transformed C3H10T $\frac{1}{2}$  and NIH MEF cells could be inhibited in a very different manner from the growth of the non-transformed parents. When C3H10T $\frac{1}{2}$  cells continued through many passages and started to lose growth control, their response to interferon eventually became very similar to that of the MSV-transformed cells (Figure 8). This suggests

that interferon affects the growth of cells by modulating a cellular growth control mechanism and that the differential effect on the growth of transformed cells was not due to MSV infection itself. At no time, however, did the C3H10T $\frac{1}{2}$  cells take on the range of transformed characteristics. Though the cells became smaller, they still retained a microfilament stress fibre system and cells remained in orderly arrays (results not shown). Furthermore, unlike the MSV-transformed cells, they remained non-tumourigenic (292).

e) Experiments with butyric acid The experiments which have been presented in this thesis employed only one dose of butyric acid, 0.5 mM, when perhaps a range of doses should have been applied to allow for different dose-dependent effects. Some preliminary tests with a range of butyric acid concentrations led to the conclusion that 0.5 mM gave clearly measurable effects without limiting the metabolic activity of the cells too severely. Butyric acid at 2.5 mM completely halted the growth of cells and was actually toxic towards them. Concentrations of 1-1.5 mM did allow cell growth, but only very slowly, whereas 0.5 mM inhibited cell growth to a clearly measurable degree, but did permit growth to continue at such a rate that full growth curves could be obtained on a similar time scale to untreated controls and interferon-treated cultures (see Figure 11).

The observed effects of butyric acid on cells were similar

to those described by others - flattening of cells, inhibition of growth but no return to density dependent growth control (8, 83, 85). The data concerning the interaction of butyrate and interferon (Figures 11 and 12), however, conflict a little with those of Bourgeade and Chany (35). They found that butyrate and interferon together were more potent growth inhibitors than the sum of the two separate agents when added to transformed cells. Butyrate was totally ineffective on normal cells and there was no synergistic effect on them. My data (Figures 11 and 12), on the other hand, show that no synergism existed in any of the cell types, rather the effects of butyrate and interferon were simply additive in all cell types, both normal and transformed.

Butyric acid inhibited growth of transformed and normal cells in much the same way. Initial growth of newly seeded cells was delayed, but logarithmic phase growth was largely unaffected, and there was minimal change in saturation density. This, coupled with the additivity, rather than synergism, of interferon and butyric acid suggests that interferon and butyric acid act by independent mechanisms.

### 3. Transformation-Specific Growth Parameters

Though the growth curves recorded in Figures 7-10 and the DNA synthesis data in Table 6 indicate that growth of normal and transformed cells may be inhibited by interferon in different ways, they alone do not confirm the existence of a

transformation-specific effect. More evidence for such an effect was obtained by studying several phenotypes characteristic of the transformed state, viz. loss of contact inhibition, anchorage dependence, raised agglutinability, rounded cell morphology and loss of microfilaments and cell surface fibronectin. This section will discuss only the first two properties and relate them to the data obtained to the effects of interferon on cloning in liquid media.

a) Focus formation All previous studies which have looked at focus formation have considered only the ability of interferon to prevent the formation of transformed foci following tumour virus infection of normal cells (71, 185). The inhibition is presumably due to the antiviral activity. This study is the first to consider interferon's ability to prevent previously transformed cells growing, due to their density independent growth, on top of a monolayer of normal cells. If MSV/MLV producer clones were used in these experiments foci could grow by virus particles spreading from the transformed cells, and infecting and transforming cells of the normal monolayer. Inhibition of focus formation by interferon under such conditions could be attributed to its antiviral activity. Non-producer clones, however, could form foci only by growth and division of the input transformed cells. Inhibition of focus formation by interferon under these conditions must be due to inhibition of cell growth.

For these reasons only non-producer clones were used in

this study. The data presented in Table 7 show quite clearly that interferon could greatly inhibit focus formation, and coupled to the data regarding cloning in liquid media suggest that this inhibition was due to a combination of interferon's antiproliferative activity and a transformation-specific effect.

b) Growth in agar Macpherson and Montagnier (173) first developed this technique to isolate transformed clones from polyoma virus-infected cells, but it was later adapted as a quantitative measure of the degree of anchorage independence of transformed clones. Gresser et al. (104) were the first to use it to measure interferon's effects on growth in agar, and since then many workers have used this technique to study interferon's antiproliferative activity (86, 103, 107, 180).

Inevitably this technique has a number of problems, principally concerning the way one measures colony number and size. Few transformed clones form colonies with 100% efficiency, and many clones form only very small colonies. There is then the problem of defining how big a group of cells must be before it can be called a colony, and defining how many colonies, as a proportion of the total number of input cells, must grow before a cell type may be called 'anchorage independent'. This was particularly crucial with most of the transformed lines used in this study since most only gradually developed anchorage independence 15-20 passages after transformation (292). There seem to be no universal criteria for defining an anchorage independent

line, and every laboratory has slightly different definitions. The ones adopted by myself, in line with others in this laboratory, were that 0.5% of the input cells should form colonies, and that a colony should be at least 0.1 mm in diameter to be recorded.

The variability in size of colonies created another problem when it came to recording colony numbers, particularly when comparing interferon-treated and control cultures. In cultures with large colonies it was possible to overlook very small ones whereas, in cultures with only small colonies, they were all counted. Interferon treatment greatly reduced the average colony size, and so it is conceivable that this could have led to an underestimate of interferon's inhibitory activity.

c) Cloning in liquid medium The measurement of interferon's effect on cloning in liquid medium was necessary in order to try to distinguish between growth inhibition and a transformation-specific activity during its inhibition of focus formation and growth in agar. Whilst it is difficult to envisage a better method to make this distinction, the belief that it is a viable method for cells in agar does rest on at least one basic assumption, namely that interferon's cell growth inhibitory activity is as effective against cells suspended in agar as against those attached to plastic in liquid medium. Bearing in mind that cells in agar and on plastic are in very different environments, and have very



different shapes which may or may not affect sensitivity to outside influences, this may be a real problem.

The effect that interferon had on colony size in agar has already been mentioned. The sizes of colonies on plastic were also reduced, in some clones (for instance MSV MEF4) quite drastically. This reduction in colony size, like colony number, was presumably due to growth inhibition, but the size reduction seen in agar colonies may in addition be due to a transformation-specific activity. These size changes were not quantitated, however, so it is not possible to fully assess their significance. Foci did not appear to vary in size - they were either present or absent.

Clearly the techniques of focus formation, growth in agar and cloning in liquid medium suffer from a number of problems as regards defining exactly what each is measuring and distinguishing between growth inhibition by interferon and a transformation-specific activity, so the exact data presented in Table 7 may be subject to some subjective bias. However, the general trends shown by the ratios of focus formation and growth in agar to efficiencies of cloning in liquid medium definitely indicate some kind of transformation-specific activity in addition to the growth inhibition.

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Glasgow et al. (86) conducted growth in agar and cloning in liquid medium together, but because no quantitative data were provided, only photographs of the Petri dishes, it was not possible to compare the two experiments. My study of growth in agar and focus formation is the first to distinguish a transformation-specific activity from interferon's growth inhibitory activity.

Firmer data about an effect on the transformed phenotype could be acquired by the study of transformation specific phenomena which may be independent of growth and thus not subject to interference by interferon's growth inhibition. Studies on agglutinability of transformed cells were early attempts to acquire such data.

#### 4. Agglutinability

The technical problems involved in these experiments were never quite overcome. For instance, even though cell suspensions were agitated constantly and the glass walls of the vessels were treated with 'repelcote' many cells still attached to them. It was very difficult to obtain consistent results, not just because of the tendency for cells to attach to the glass, but also because an aliquot removed from one part of the suspension often contained widely differing concentrations of aggregated cells from those in an aliquot removed from another part of the suspension.

Although a reduction in agglutinability in interferon-

treated cultures was observed on several occasions, these results were very variable, and so should be treated with caution. Nevertheless, these studies could be improved and firmer data obtained.

## 5. Adhesiveness

The studies on adhesiveness of cells show quite clearly that interferon had no significant effect on this parameter. The aims of these experiments were to assess interferon's effect on cell adhesivity per se, and the ability of the cells to respond to serum in the medium (which contains fibronectin). Other workers have found that presoaking the substratum with serum or fibronectin aids the adhesion of cells which do not produce their own fibronectin when a cell suspension is subsequently added to the substratum (126, 199). Thus in my experiments glass vials were presoaked with PBS or fibronectin, and cell suspensions were kept free of serum. Perhaps it would have been more consistent with the rest of this study had serum been present in or absent from the cell suspension and prior soaking of the glass omitted.

## 6. Morphology and Fluorescence

### a) Experimental technique

(1) Microscopy Microscopical studies can give misleading results due to the large degree of subjective judgement that is required, and the difficulty in making quantitative

measurements. The problem of subjectivity is especially acute at high magnifications where such a limited field of view is visible at any one time that it is very easy to select fields that are not representative of the culture as a whole. Studies with crystal violet-stained cultures were generally free of this problem since very low magnification was used to provide an overall image of cell and culture morphology.

With fluorescence microscopy, however, it was a problem, and there was often a temptation to photograph fields which contained especially striking patterns, even though they may not have been entirely representative of the culture as a whole. Nevertheless this temptation was always avoided and the photographs presented in Figures 13 to 16 are representative.

Coupled to this problem of representativeness is the problem of quantitation of observations. In Figure 13, interferon-treated cells are quite clearly flatter than control cells, but the methods that were available for assessing what proportion of cells in the entire culture responded in that way, and by how much cell spreading was increased could give only partial answers. A rough guide, used in this study, was to measure the range between minimum and maximum lengths of the longest dimension of a large number of cells in each culture. A more satisfactory method may have been to trace the outlines of cells onto graph paper and record the number of squares each cell covered. Such a determination might give a more accurate indication of the

extent of increased cell size, but would add little more to the obvious observations from Figure 13 that interferon treatment has enlarged the cells.

Quantitation could yield useful additional data when high magnification microscopy is used, due to the limited field of view.

(ii) FITC-DNase staining Quantitation was attempted with FITC-DNase stained cultures of MSV C3H2, where cells in different fields were scored for presence or absence of microfilament bundles. This was not attempted with MSV MEF2 since, due to the high background staining, it was quite difficult to be certain whether or not cells contained microfilament bundles.

Whilst background staining varied between different cell types for any one batch of FITC-DNase, the ability to stain microfilament bundles also appeared to vary for different batches on the same cell type, thus calling into question the efficacy of this staining method. In general this technique did appear to be a satisfactory alternative to the use of antiserum to actin (which had proved difficult to prepare due to poor antigenicity of actin), and in a study to compare the efficiency of anti-actin, DNase I plus anti-serum to DNase I, and FITC-DNase I for staining microfilaments, Wang and Goldberg (260, 261) found that FITC-DNase I compared quite favourably, even with anti-actin. The variability I experienced with different batches may have been due to

contamination with unconjugated FITC, despite gel filtration to remove unbound FITC. Free FITC and DNase could be generated in a batch of FITC-DNase during storage if the bond were unstable at  $-20^{\circ}\text{C}$ . This was not assessed, but it is known to be unstable at  $4^{\circ}\text{C}$ .

(iii) Anti-fibronectin staining The studies conducted on distribution of cell surface fibronectin showed that interferon had no effect, unless butyric acid was also present. These studies are, however, incomplete since the effects of interferon on fibronectin beneath the transformed cells were not looked at, only the distribution across the upper cell surfaces.

The main difficulty that arose with these experiments was that on a number of occasions there was some variation in cell density at the time of fixation, which could influence differences in fibronectin patterns in a way similar to those observed by Mautner and Hynes (1977). They found that the intercellular matrix of fibronectin fibres developed and became increasingly complex as cultures of normal cells approached and attained confluence. Furthermore, multiple layers of cells in transformed cultures sometimes made it difficult to focus on the interwoven mesh of fibronectin fibres.

(iv) Quantitation of actin and fibronectin in cells Initially, attempts were made to assess not only the total quantity of actin present within cells, but also the percentage polymerised into microfilaments. This was studied using an

assay developed by Blikstad et al. (32) which took advantage of the fact that monomeric actin would inactivate DNase I whereas polymerised actin would not. Unfortunately in my hands this assay method would not work reliably. There have also been no more studies published which have used this assay.

The polyacrylamide gel electrophoresis subsequently employed was able to give an indication of total actin present in cells, but of course would not distinguish between monomeric and polymerised actin. The use of  $^{35}\text{S}$ -methionine meant that only proteins containing this amino acid were visible on autoradiographs, and the short labelling period (45 minutes) tended to measure rate of synthesis of methionine-containing proteins rather than the actual amount of protein present. This may explain the contradiction between Figure 20c and Figure 15c (iv) where butyric acid and interferon treatment together appeared to have little effect on MSV C3H6 cellular fibronectin levels, according to gel electrophoresis, but clearly greatly increased its organisation and probably total amount present on the cell surfaces, according to immunofluorescence. This apparent conflict could be reconciled if butyric acid and interferon had no effect on rate of fibronectin synthesis, but was able to stabilise fibronectin at the cell surface, reducing its turnover and loss to the medium.

b) Interpretation of microscopy studies The main question mark over these morphological studies is whether the observed



effects were a manifestation of a transformation-specific property of interferon. Clearly interferon-treated transformed cells looked more normal, especially if butyric acid was also present. However, if the morphological changes were a reverse transformation, why were the morphologies of C3H10T $\frac{1}{2}$  and C3H MEF also affected? For C3H10T $\frac{1}{2}$  it could be that, being an established line, it was already partially transformed, i.e. in a pre-malignant state, which was also reversed by interferon. This explanation is unsatisfactory for C3H MEF cells, however, since these cells were completely normal, having been freshly taken from embryos. Pfeffer *et al.* (201) also observed that interferon treatment of human embryonic fibroblasts resulted in increased cell spreading and an increase in cell volume and surface area. They noted that decreased growth rate often correlated with these morphological changes, and they drew parallels between interferon-treated early passage fibroblasts and late passage senescent fibroblasts. They suggested that interferon may render the cells more sensitive to a normal growth control mechanism. Certainly C3H MEF fibroblasts also increased their size and degree of spreading during senescence. The lack of change of NIH MEF cell morphology in response to interferon may have been due to general insensitivity towards interferon, or perhaps these cells may have already entered senescence when treated with interferon, rendering them resistant to interferon, or masking any effects that interferon might have had. (It is quite

possible that NIH MEF cells were entering senescence during these experiments since secondary cultures were generally used, and cells at the third passage clearly were senescent).

Fuse and Kuwata (78) also linked morphological changes with decreased growth rate with their suggestion that transformed human RSB cells, treated with interferon changed from a fibroblastic to an epithelial morphology due to the cells being halted in S phase. The interferon-treated transformed cells in Figure 3d (ii), e (ii) and f (ii) are indeed more epithelial in appearance than the controls in Figure 13 d (i), e (i) and f (i), but the explanation proposed by Fuse and Kuwata for this morphology change seems unlikely. They claimed that growth of interferon-treated cells was blocked in S phase, but many studies have indicated that at least the main block for both normal and transformed cells is in  $G_1$  (170, 194, 231, 264), though some have found an additional block in  $S+G_2$  (18, 54, 79, 175).

Subsequent studies by Pfeffer et al. (202) found that the interferon-treated human fibroblasts showed an increase in intracellular microfilament bundles and cell surface fibronectin fibres. My studies, on the other hand, indicated that interferon had no effect on C3H MEF fibronectin distribution. Others (174, 247) have recently found that interferon may increase the microfilament bundles in transformed cells, in agreement with the results presented here (Figure 14). Whilst it is conceivable that the changes observed by Pfeffer et al.

(202) were the result of a slower growth rate, this is unlikely to be the explanation with the transformed cells, at least in this study, due to the limited effect that interferon had on growth rate of subconfluent cells (Figure 7).

The decrease in total cellular actin (Figure 19) and fibronectin (Figure 20) upon transformation concur with the findings of a number of studies (70, 215, 274), though others have found that actin content may remain unaffected by transformation (ref 128 p88). Despite the increases in frequency of microfilament bundles, no consistent changes in total cellular actin or fibronectin in interferon - or butyrate-treated transformed cells were observed. If interferon did cause reverse transformation it might be thought that it may be accompanied by a return of actin levels towards those seen in the C3H10T $\frac{1}{2}$  cells, but this does not necessarily need to be so. As already mentioned, the apparent conflict between the immunofluorescence results of MSV C3H6 cells treated with interferon and butyrate together, and the gel electrophoresis results may be reconciled, by proposing that this treatment has no effect on rate of synthesis of fibronectin, but decreases its turnover.

It is perhaps surprising that though interferon treatment appeared to increase the frequency of microfilaments, there seemed to be little change in the fibronectin pattern. Virtually all studies to date that have studied both microfilaments and fibronectin have found that changes in one

result in parallel changes in the other (128, 131, 177, 274). Since both appear to be important in controlling cell shape and adhesion, the increased cell flattening presumably should require increases in levels of extracellular matrix as well as the intracellular microfilament system. The lack of change in the fibronectin matrix could be explained if another protein, such as collagen, were involved in the spreading of these cells. One point against this possibility is the findings by Chen et al. (49) that the extracellular matrix of chick embryo fibroblasts and myoblasts normally consists almost entirely of fibronectin and no collagen. However, these studies were not extended to include transformed mouse fibroblasts, and the possibility cannot be ruled out that synthesis of a new cell surface protein may be activated, though the polyacrylamide gels (Figure 18) argue against such a possibility.

The fact that the transformed cells studied did possess a little fibronectin, may mean that enough matrix existed for the cells to spread out to the degree stimulated by butyrate or interferon alone, without the need for new matrix to be laid down. The increased fibronectin on the surfaces of transformed cells under the influence of the two agents together may have been a response to a demand for the cells to flatten to a much greater degree than the existing matrix could allow. On the other hand, the increased flattening could be due to some cooperative activity between butyric acid

and interferon, despite their diverse effects on growth rate; e.g. increased intercellular communication and contact inhibition. This might then enable the transformed cells to lay down and retain a more complex matrix.

c) Possible role for interferon in control of growth rate by cell shape Some correlations between interferon-induced morphological changes and decreased growth rate have already been mentioned. The field of morphology and growth control has been most closely studied by Folkman and his colleagues (72, 73, 41). They proposed that in normal cells growth rate and cell shape were closely linked. Thus growth could be maintained only if the cells remained flattened. As a result, at confluence, as cell crowding increases and each cell becomes more rounded, DNA synthesis slows and eventually stops. Such control may in part be a result of a limitation of intracellular supply of nutrients. Increased flattening of serum-starved and senescent cells may be due to increased sensitivity to this control process. This growth control mechanism may be the same as that proposed by Pfeffer et al. (201, 202) to explain the inhibition of growth and increased cell size of human fibroblasts by interferon. This too would explain the increased flattening of C3H MEF and C3H10T $\frac{1}{2}$  cells. In this respect it is interesting that increasing the serum supply can induce cell rounding and overcome growth inhibition induced as a result of cell crowding, serum starvation,

senescence (though only initially) and interferon treatment, suggesting that serum can at least partially ease restrictions of the control process.

Folkman and Moscona (73) proposed that the reason that transformed cells were able to grow regardless of their shape may be due to loss of sensitivity to this control mechanism. If this is so then the data presented in this thesis go some way to suggesting that interferon may at least partially recouple growth control and cell shape, thus explaining both reduced saturation density and cell flattening. On the other hand it is conceivable that interferon could impose a substitute control mechanism to compensate for the breakdown of the normal process.

My data would also suggest that loss of sensitivity to this growth control mechanism may not correlate fully with MSV transformation. For instance, MSV C3H2 cells appeared to retain the control for several passages after transformation (Figure 7), while C3H10T $\frac{1}{2}$  started to lose this sensitivity at later passages (Figure 8). These two examples also clearly illustrate how interferon's effect changed depending on whether or not they were sensitive to growth control.

Recently Brouty-Boye et al. (40, 41) were able to stimulate transformed C3H10T $\frac{1}{2}$  cells (transformed by X-irradiation) to revert to the normal phenotype by prolonged culturing at very low density on plastic on which they were able to maintain a well-spread form. These revertants also showed

morphology-linked growth control as demonstrated by growth on various thicknesses of poly(HEMA) (41). These experiments raise the possibility that prolonged interferon treatment of transformed cells, which show increased flattening as a response to interferon, may eventually result in formation of stable transformation revertants. This has, in fact, been shown to be the case by Chany and Vignal (48) who found that interferon treatment for 200 passages of a producer clone of MSV-transformed BALB/c 3T3 cells eventually resulted in the formation of stable revertants. It is possible, however, that such an effect may have been due, at least partly, to an antiviral activity.

#### 7. Effects of Interferon on p21 Levels

a) Experimental techniques The experiments to measure levels of intracellular p21, the putative transforming protein of KiSV, suggest that interferon treatment might have lowered them somewhat, by degrees roughly in line with the observed relative sensitivities of each transformed clone towards interferon. However, the techniques used to quantitate the amount of p21 in gel bands are subject to so many variables that it would be difficult to draw firm conclusion from changes which were after all, relatively small. Such variables include the losses during the preparation of cell extracts, accuracy of locating and cutting out the p21 protein on the gel, the efficiency with which the gel scintillant solubilised the

protein, the variability of methionine and total protein content of control and interferon-treated cultures, and the variability of amounts of proteins 1, 2 and 3 (with which p21 was compared) in control and interferon-treated cultures.

The variability of total protein was partly overcome by relating the cpm of p21 to the total protein per culture dish as determined by the Lowry assay. However, perhaps a more meaningful comparison may have been to relate the p21 cpm to the total protein content of the number of cells applied to the gel.

b) Relevance to other studies It has been proposed that pp60<sup>src</sup> of Rous Sarcoma Virus may transform cells by acting directly on the cytoskeleton (66, 178, 179) or some cell surface components (129, 154). Indeed since pp60<sup>src</sup> is now known to be concentrated in the plasma membrane (129), it raises the possibility that it may be ideally located to somehow induce the severance of the putative links between intracellular microfilaments and extracellular fibronectin. One possible means may be to activate a proteolytic enzyme by phosphorylation. This could cause the disaggregation of the microfilaments and shedding of the fibronectin, resulting in at least some properties of the transformed phenotype becoming manifest. In addition, or alternatively, pp60<sup>src</sup>'s location may alter in some other way cellular communications between the internal and external environments, which may hinder growth control, for instance. The cellular equivalent



pp60<sup>sarc</sup>, which is produced in normal cells in much lower quantities than is pp60<sup>src</sup> in transformed cells, is thought to be a normal growth regulator (see Introduction), so it may well be that large amounts of pp60<sup>src</sup> may upset growth control. It is much less clear how the cellular activities and location of p21 of Kirsten Sarcoma Virus-transformed cells compare with those of pp60<sup>src</sup> (89), nor even if it is actually the transforming protein of KiSV and HaSV. If p21 is the transforming protein, then reverse transformation may be induced by interferon by a number of effects on it. The data presented in these experiments indicate that interferon probably did not inhibit the synthesis of p21, but it may still block its activity or have some effect on the target of p21 activity, e.g. growth control.

### III. General Conclusions

The aim of this thesis was to establish whether or not interferon could revert the phenotype of transformed cells towards more normal behaviour. Of the data presented no single experiment has proved the existence of such a reverse transformation activity for interferon. However, overall the evidence argues very strongly in its favour (118). The strongest evidence comes from the studies on focus formation and growth in agar. Interferon clearly inhibited these transformation-specific phenomena to a much greater degree than

the antiproliferative activity inhibited cloning in liquid media.

The growth curves (Figure 7) provide slightly less convincing evidence since other explanations for the reduced saturation density of transformed cultures were possible. However, increased density dependent growth control is still the most likely explanation.

The morphological studies showed that interferon treatment did increase cell spreading, and could instigate at least a partial restoration of the microfilament bundles of transformed cells. These observations are consistent with reversion of the transformed phenotype, though since C3H MEF cells also spread out further, there must be some doubt.

There is evidence that growth rate and cell morphology are closely linked in normal cells by a common control mechanism from which transformed cells may have escaped. Interferon's effect on normal cells may be to increase their sensitivity to this regulatory mechanism, perhaps in a similar way to serum starvation, density dependent growth control and senescence. Its effects on transformed cells may be to reimpose this control mechanism, so that growth rate and morphology are once again linked. If this is so, then interferon's growth inhibitory activity and reverse transformation activity may not be so very different. The former would make normal cells more sensitive to the control, and the latter reimpose that control on transformed cells.

How interferon might exert this effect on transformed cells is not known. It appears that, in these KiSV-transformed cells at least, it does not act by inhibiting the synthesis of the putative transforming protein, p21.

Obviously these proposals must be backed up by firmer experimental data, for instance by conducting studies into interferon's effects on growth of different shaped cells on poly(HEMA), as described by Folkman and Moscona (73).

Further data to characterise the reverse transformation activity could be obtained by studies on phenomena which are not growth-related, such as secretion of proteases (note the inhibition of secretion of plasminogen activator (223) ) and other transformation-specific proteins (88, 226). The preliminary studies on agglutinability conducted should also be improved and extended.

Of more immediate significance to clinical application are the questions of just what degree of reversion may be possible, and how stable it may be after cessation of a prolonged interferon treatment.

Studies over the past few years established that interferon probably could exert its antitumour activity in vivo by two mechanisms - firstly by a direct effect that inhibited the growth of the tumour cells, and secondly by an indirect effect which stimulated the activity of the immune system, particularly macrophages, T-lymphocytes and Natural Killer cells (84, 94, 103, 105, 112, 114, 183, 184). A number of studies

have been undertaken to determine whether one of these activities in general plays the dominant role in vivo (93, 103, 105, 246, 281). Most experiments that have studied the effects on interferon on the growth of tumour and transformed cells, both in vivo, and in vitro, have considered that only the antiproliferative activity is responsible for inhibiting tumour and transformed cells' growth (86, 151, 190, 194, 201, 254). The experiments conducted in this project have shown, however, that interferon can prevent growth of transformed cells by an activity which reverts the behaviour of the cells towards a more normal phenotype.

The identification of this third possible antitumour mechanism underlines the diversity of interferon's metabolic activities, and increases the likelihood that a direct effect on tumour cell behaviour plays an important role in interferon's antitumour activity. Thus, this project has increased our understanding of the way in which interferon exerts some of its cellular effects, and has greatly strengthened interferon's position as a potentially powerful antitumour agent.

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